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Determining Trace Levels Of Tetracycline In Solid And Liquid Samples Utilizing Complexation With A Transition Metal Cation

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ABSTRACT

Determination of tetracycline is achieved by incorporation into an octahedral complex with the transition metal cation iron(III), producing a red-brown mixture. An absorption spectrum, of the iron(III)-tetracycline complex shows an absorption maximum at 435 nm, which was monitored for determination by spectro-photometer. The complex formed was stable for more than 48 hours at room temperature. A spot test assay was found to be sensitive to less than 30 mg of the antibiotic. An interpolative assay which is performed in micro-reaction tubes was able to detect amounts to less than 30 μg of tetracycline. A microvolume assay for microliter samples of tetracycline (volumes less than 30 μliters) demonstrated sensitivity to as little as 6 μg tetracycline. Determination by standard curve allows rapid analysis of multiple samples and is shown linear over a sixteen fold range of concentration from 2.813×10^{-5} molar to 4.501×10^{-4} molar erythromycin, ampicillin, and streptomycin, which are common antibiotics, do not interfere with the assay results and can be present. Inorganic salts such as phosphates, azides, chlorides, carbonates, and bromides do not interfere with the assay result for tetracycline. Medicinal compounds such as aspirin, sulfanilamide, and quinine did not cause interference. The colorimetric response is easily discerned. Key Words: Tetracycline; Iron(III); Colorimetric; Assay, Spot test. © 2007 Trade Science Inc. - INDIA

KEYWORDS

Tetracycline;
Iron(III);
Colorimetric;
Assay;
Spot test.

INTRODUCTION

Tetracycline is a broad spectrum antibiotic which is widely used. The annual industrial production of tetracycline is among the highest of all antibiotics. Toxic exposure in industrial environments is known to be a causative factor for significantly altered serum immunoglobins[1]. Microbial resistance to tetracycline is a growing problem, often due to the acquisition of new genes by bacteria[2]. Tetracycline utilized in farm animals such as calves[3], household pets[4], swine[5], horses[6] and chickens[7]. Previous studies have discerned problematic results of tetracycline addition to animal feeds[8]. Tetracycline is widely used as an additive to feeds to achieve improved growth rates in calves and swine[9]. The utilization of bovine and swine bones for bone meal foodstuffs from animals raised with tetracycline usage have been of problematic[9]. Tetracycline residues have been shown to exist in eggs obtained from exposed chickens[10]. Tetracycline residues have been found in milk derived from exposed dairy cows[11]. These concerns have led to the appearance of analytical methods of determining antibiotics in milk, eggs, and meat[12]. Studies have shown excretion of tetracycline from usage in humans derived from capsulated formulations[13].

The tetracycline group of antibiotics is considered to be bacteriostatic[14,15], and inhibit microbial protein synthesis[15]. Tetracycline is a broad spectrum antibiotic[16], which inhibits almost all gram positive and gram negative bacteria[16]. It has the basic structure of a naphthacene ring system[16], and inhibits eubacteria, obligately parasitic bacteria[15] and chlamydia infections[17,2]. Tetracycline blocks the binding of the amino acyl-tRNA to the A-site (amino acyl-tRNA binding site)[15, 18, 19]. In this action, the tetracycline molecule can bind to either the large or the small ribosomal subunit and creates an unstable binding to aminoacyl t-RNA[20].

The red-brown colorimetric response occurs when iron(III) forms the characteristic octahedral complex with phenolic hydroxyls[21]. Previous studies indicate that one iron(III) cation will assemble six molecules of a monohydric phenolic species to form the complex[22]. A strong colorimetric response is observed and the observed hue can vary according to conditions of concentration, solvent type, and time of reaction[23].

Monitoring tetracycline contamination of the environment will assist in identifying the sources as biological or industrial. The colorimetric assays which are presented here should be useful for applications in areas of clinical analysis, industrial manufacturing, and environmental monitoring. The methods presented here are fast, simple, specific, and sensitive to microgram amounts of tetracycline.

EXPERIMENTAL

Chemicals and apparatus

Reagents were obtained from Aldrich Chemicals, P.O. Box 14508, St. Louis, MO. 63178, USA. A Perkin Elmer Lambda 6 U.V.Visible dual beam spectrometer and a Spectronic 21D visible spectrometer were used for absorption measurements.

Spot test assay for tetracycline

In plastic or glass reaction tube vessels place 50.0 micro-liters (μL) of aqueous 1.0% $FeCl_3$, then 100 μL of CH_3OH, followedby up to 50.0 μL of aqueous or methanolic test sample. Color develops in one minute, and compare to a known tetracycline positive control and negative control having no tetracycline. Finaltotal volume of mixture becomes 200 μL.

Colorimetric interpolative microassay

In micro reaction tubes, place 110 microliters (μL) of a sample dissolved in methanol. Add 10.0 μL of aqueous 1.0% $FeCl_3$ (final total volume becomes 120.0 μL). Color develops in < 1 minute.Compare to a set of prepared tetracycline sample controls having 30 μg to 120 μg of tetracycline. A stock solution of tetracycline at 3.00 mg/mL is convenient for generation of necessary controls. Unknown test samples can be quantified by interpolative comparison to known controls. Control concentrations found useful are as follows (micrograms of tetracycline): 15, 30, 60, 120, 180.

Quantitation of tetracycline for microvolume samples

In micro reaction tube vessels suitable for 20.0μL

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volume, place up to 15.0 μL of dissolved unknown and 5.0 μL of aqueous 1% $FeCl_3$ (final total volume is 20 μL). Allow one minute for color development. A six microgram quantity of tetracycline can be detected. A known tetracycline sample is utilized for a positive control and a negative control having no tetracycline.

Standard curve development for multiple assays of tetracycline

In a suitable vessel place 4.00 mL of CH_3OH, 1.50 mL of aqueous 1.0% $FeCl_3$, and up to 0.50 mL of sample dissolved in water or methanol (total volume becomes 6.00 mL). Use a stock solution of tetracycline at 3.00 mg/mL (in methanol) to make a set of known samples from 100 micrograms to 1200 micrograms tetracycline. Allow five minutes for color development. Read in U.V.Visible spectrometer at 435 nm wavelength for absorbances. Test samples are treated similarly.

Background evaluation of organic and inorganic compounds

Following the protocol given for spot test assay, 0.1 mg of the inorganic salts and organic compounds listed in TABLE 1 were individually placed with 0.1 mg of tetracycline. The spot test results showed clearly the diagnostic red-brown solution indicative of tetracycline with no interfering signal.

Sample collection and testing for environmental contamination

A one milligram amount of tetracycline was ground in mortor and pestle then randomly dispersed over a 12 inch square portion of table top. Then a clean sterile cotton tipped applicator stick was used to collect dust particles be using a wiping motion back and forth across the foot square zone of desk top.

The cotton tip was washed with absolute methanol into a test tube, allow to dry by air flow. Then the spot test protocol was followed to determine the presence of tetracycline in the collected samples.

Various solid and liquid samples (< 0.50 mL volume) of tetracycline were made and assayed accordingly by the standard curve protocol at wavelength of 435 nm. Various medicinal drugs were combined with known amounts of tetracycline to test for interferences.

RESULTS AND DISCUSSION

The transition metal iron (Fe) in its oxidation state of +3, will readily form a colored complex with compounds containing phenolic functional groups. When following the procedures described here the colored iron(III)-tetracycline complex will form quickly and remain stable for more than 48 hours when kept at room temperature. The reddish-brown appearing mixtures are a result of the formation of a metallo-organic complex[21]. The iron and analyte complex formation is quantitative and allows detection of microgram amounts of analyte. The structure of tetracycline is shown in figure 1 and shows the position of the phenolic hydroxyl group, aromatic ring, and amide group.

Complexation of the tetracycline molecule to iron(III) is accomplished through attractive forces of the phenolic group. The monophenolic analytes are in complex with the cation iron(III)[23]. A double beam Perkin Elmer U.V.-Visible spectrometer was utilized to acquire the absorption spectrum of the iron(III)-tetracycline complex from wavelengths 374 nm to 700 nm (see Figure 2). Under these solution conditions a strong absorption maximum occurs at 435 nm which was used for quantitative assay.

The protocol for microvolume assay provides a means to quantitate tetracycline in microliter volumes and produces a final volume of 20 mL of solu-

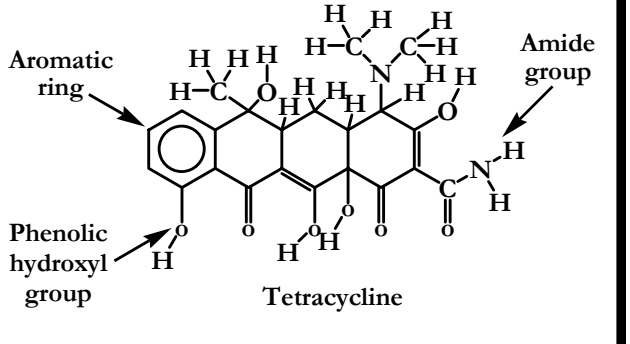

Figure 1: Molecular structure of tetracycline is shown with positions of the phenolic hydroxyl group, amide group, and general structure features indicated

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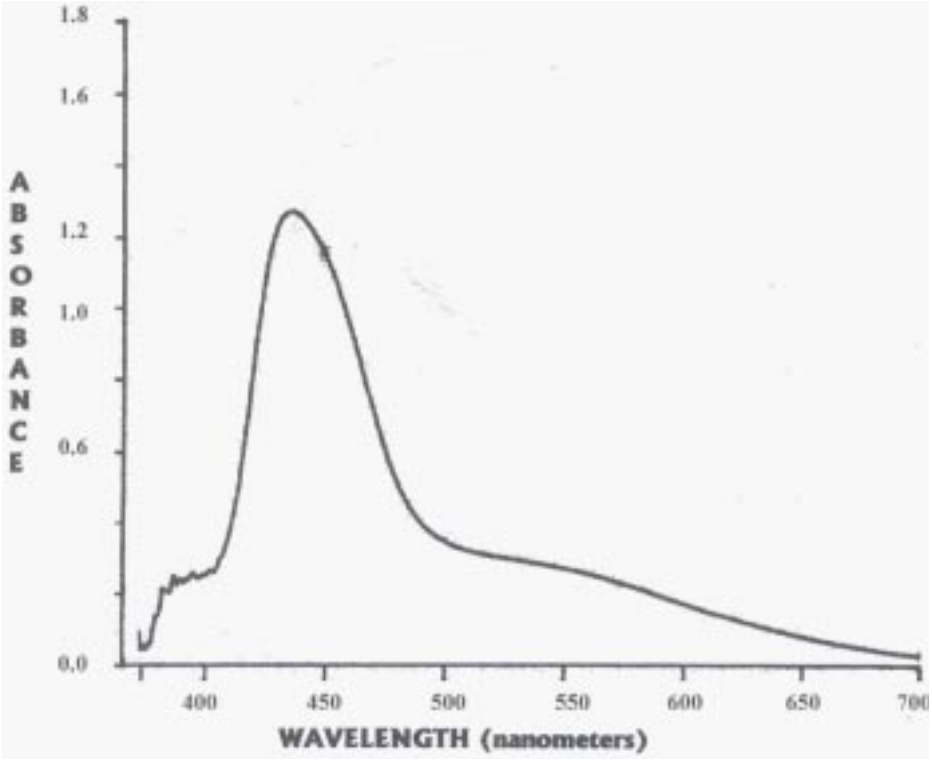

Figure 2: Absorption spectrum of the iron(III)-tetracycline complex is shown as absorbance versus wavelength (nanometers) from 374 nm to 700 nm. The absorption peak at 435 nm was utilized for assaying tetracycline by use of a standard curve (see Experimen- tal)

tion. The maximum working sample volume is 15 μL and is sensitive for as little as 6 μg tetracycline.

A positive control is run simultaneously with the test sample and should have a known amount of tetracycline or other monohydric phenolic compound. A stock solution of 3 mg/mL of tetracycline is a convenient concentration for preparation of controls and standards. A negative control sample containing no tetracycline is run simultaneously. Antibiotics erythromycin, streptomycin, and ampicillin are also very common and will not complex with iron(III) and will show a negative result (no color formation), when following these procedures. Glass or plastic containers of appropriate size were found to function well with the spot test or microassay protocols with no caustic gases.

The spot test assay for tetracycline demonstrated sensitivity to less than 30 μg of analyte. The red-brown color of the mixture was again strong and easily discerned (see Figure 3). The antibiotics ampicillin, streptomycin, and erythromycin, shown in figure 3 at positions A, B, and C respectively, did not complex with iron(III) and demonstrate a negative response. In addition, these results are comparable to the response with the negative control, position G, of benzoic acid. Ampicillin, streptomycin, and erythromycin are widely used antibiotics, but do not have a phenolic functional group. Other nonphenolic antibiotics should likewise not develop the red-brown colored endpoint via iron(III).

TABLE 1 lists the inorganic and organic compounds which do not produce background color formation when present with tetracycline in samples collected for analysis.

Inorganic salts such as carbonates, chlorides, bromides, and phosphates which are ubiquitous can be present with tetracycline during the assay procedure. The list is not exhaustive and will include other medicinal compounds of clinical importance and not having phenolic hydroxyl groups. Salicylic acid was an efficient positive control, having a monohydric phenolic hydroxyl group.

The interpolative microassay allows determination of tetracycline by comparing to control samples

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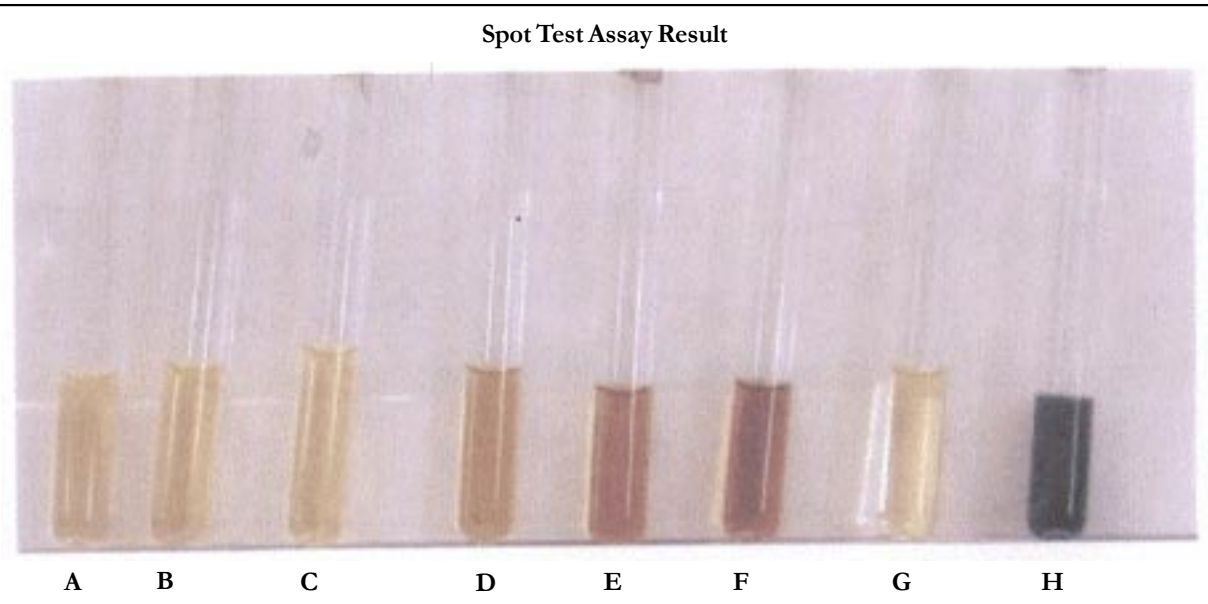

Figure 3: Spot test assay results demonstrating the red-brown color development of the iron(III)-tetracycline complex. Samples shown are as follows: A) Ampicillin at 60 μg; B) Streptomycin at 60 μg; C) Erythromycin at 60 μg; D) Tetracycline at 30 μg; E) Tetracycline at 60 μg; F) Tetracycline at 120 μg; G) Benzoic acid at 60 μg as Negative Control; H) Salicylic acid at 60 μg as positive control

TABLE 1: Non-interfering compounds

Organic Compounds	Inorganic Salts
Erythromycin	$CaCO_3$
Streptomycin	NaCl
Ampicillin	NaN_3
Aspirin	LiBr
Benzenesulfonamide	K_2HPO_4
Sulfanilamide	$Na_2B_4O_7$-$10H_2O$
Quinine Sulfate	Na_2CO_3
Caffeine	NaH_2PO_4
p-Nitrobenzoic Acid	-
Maleic Acid	-
Myristic Acid	-
p-Tartaric Acid	-
Sulfamic Acid	-

having known amounts of the analyte. In addition, it is sensitive to less than 30 μg of analyte (see Figure 4). Streptomycin and erythromycin again do not form the positive red-brown response nor any other interfering background signal. The test sample (sample A of Figure 4) has 75 μg of tetracycline, and produces a red brown end point that clearly places it between sample E (at 60 μg of tetracycline) and sample F (at 90 μg of tetracycline). In this manner, using interpolative examination, the amount of tetracycline in an unknown sample can be estimated. The negative control, which is sample H (benzoic acid), does not form a red-brown. The positive control at position I which is 30 μg of salicylic acid forms a stark blue colored mixture. Sample D of figure 4 demonstrates the least concentration of tetracycline at 30 μg, but color formation is still clearly discerned when compared to the negative test samples such as streptomycin and erythromycin (samples B and C, respectively), and the negative control sample H (Benzoic acid). These findings demonstrate the significant levels of sensitivity and specificity possible with the simple and rapid protocols presented here.

Analysis of numerous and continuous unknown samples can be examined by construction of a standard curve of which an example in figure 5 is shown. The use of a standard curve allows the examination of large numbers of unknown test samples and is sensitive to amounts of tetracycline of less than 75 μg in six milliliters. The procedure described in Experimental produces sample mixtures having a final total volume of 6.00 mL.

The plot presented in figure 5 demonstrates a set of standard solutions which range in concentration of analyte from 12.50 μg/mL to 200.0 μg/mL tetracycline. The correlation coefficient by linear re-

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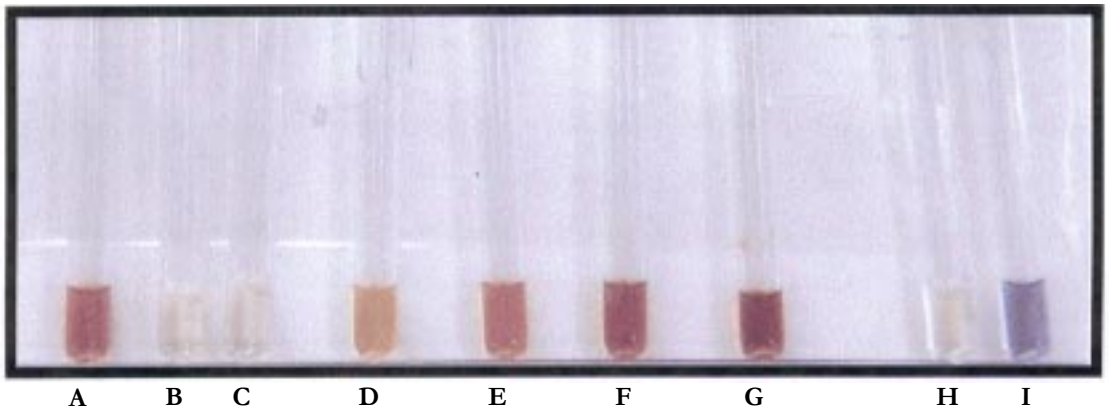

Figure 4: Colorimetric interpolative micro assay results showing red-brown color of the iron(III)-tetracycline complex. Samples shown have the following amounts of compounds as indicated: A) Test sample having 75 μg Tetracycline; B) Streptomycin at 60 μg C) Erythromycin at 60 μg; D) Tetracycline at 30 μg; E) Tetracy- cline at 60 μg; F) Tetracycline at 90 μg; G) Tetracycline at 120 μg; H) Benzoic acid at 30 μg as negative control; I) Salicylic acid at 30 μg as positive control

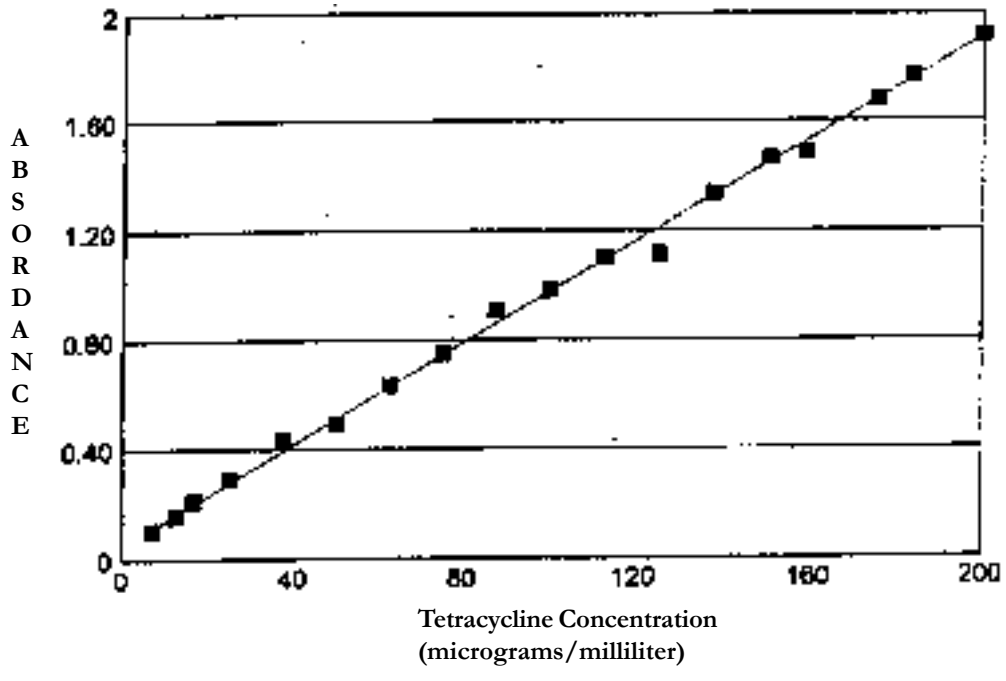

Figure 5: A standard curve demonstration for multiple sample determination at wavelength of 435 nm. Correlation coefficient of line constructed is 0.9980, the slope of line shown is 1.540E-3 milliliters/ microgram and y-axis intercept is 0.05669

gression was calculated to be 0.9980 for the graphed line, showing high correlation. The slope of the line shown is 0.001540 milliliter/microgram (Y-axis intercept is 0.05669). All test samples were examined at a wavelength of 435 nm utilizing a U.V.Vis. spectrometer. A stock solution of tetracycline at 3.000 mg/mL works efficiently for producing standard solutions in this concentration range.

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The molecular structures of ampicillin, erythromycin, and streptomycin are shown for comparison in figure 6. Functional groups present include amides, amines, hydroxyls, esters, cyclic amides, and carboxylic acids, demonstrating a broad range of organic functional groups that do not form interfering signals. The requirement of a phenolic hydroxyl group for this colorimetric complexation reaction provides a high level of specificity for tetracycline.

The results of an interpolative microassay are presented in figure 7. Fifteen micrograms of tetracycline is dissolved into the lowest concentration con-

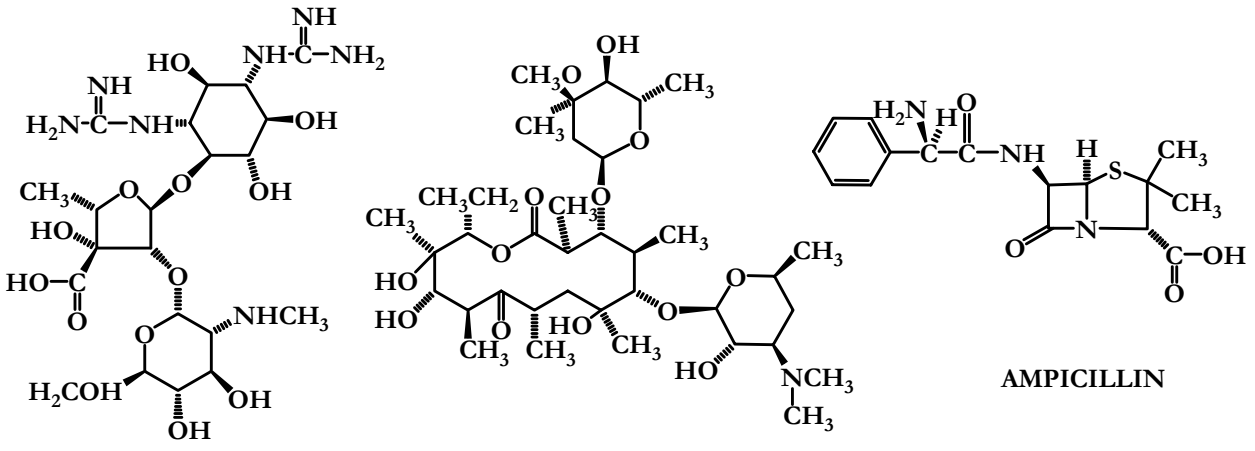


Figure 6: The molecular structures of ampicillin, streptomycin, and erythromycin. No phenolic hydroxyl groups exist in these molecules and these antibiotics may be present simultaneously during the determination of tetracycline

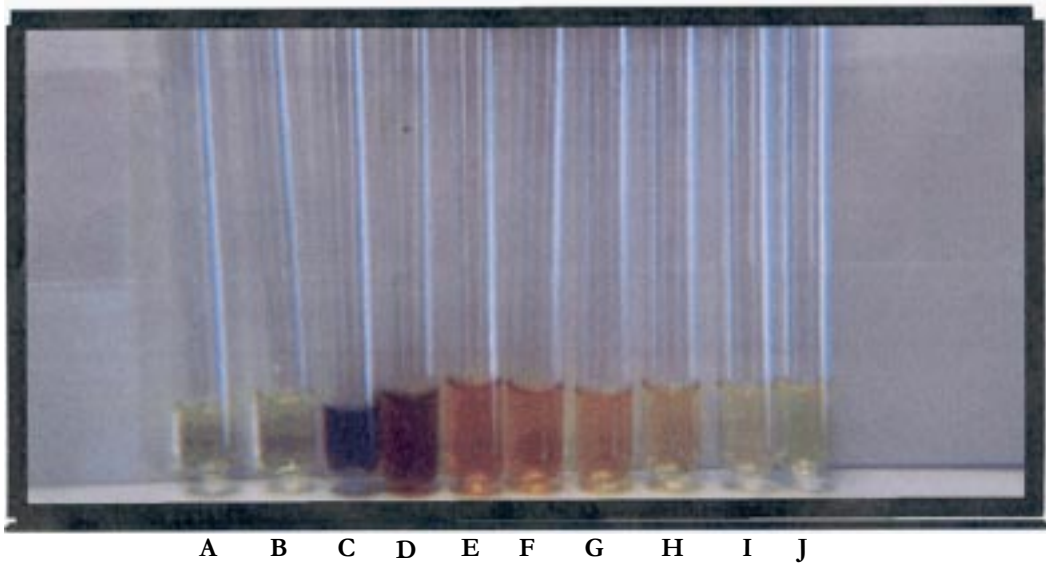


Figure 7: The interpolative microassay of tetracycline at various concentrations which shows the strong colorimetric results. Samples are: A) Erythromycin at 30 μg; B) Streptomycin at 30 μg; C) Salicylic acid as positive control at 30 μg; D) Tetracycline test at 120 μg; E) Tetracycline control at 60 μg; F) Tetracycline test at 45 μg; G) Tetracycline control at 30 μg; H) Tetracycline control at 15 μg; I) Tetracycline test at 3 μg; J) Blank as the negative control, no compounds are present

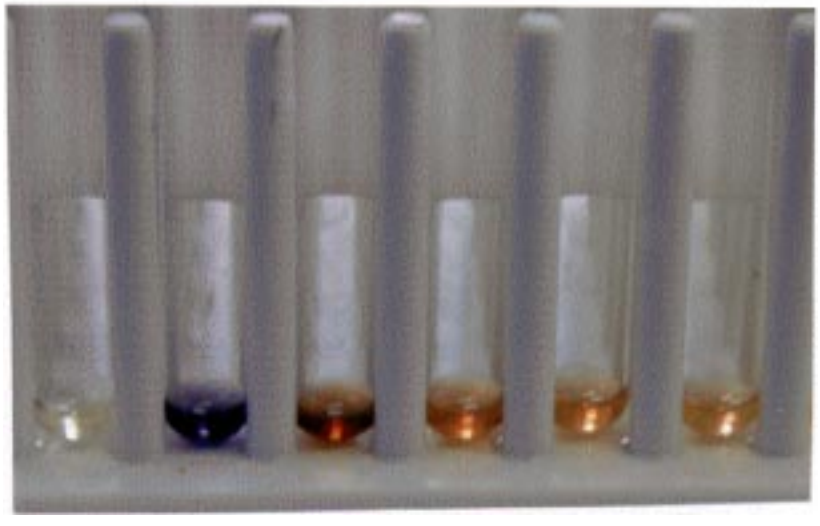
trol which itself is easily discerned (see Figure 7). Also observed is the lack of interference from the antibiotics erythromycin and streptomycin, each being at a concentration of 30 μg in 120 μL volume. The positive control in this example is salicylic acid, however other simple monophenolic organic compounds will function in this manner. The actual test samples are D, F, and I which clearly fall within the colorimetric results of the control samples which are E, G, and H (see Figure 7). In this manner, a fast and simple approach can be used to obtain a useful approximation of tetracycline concentration.

To demonstrate the efficacy of the spot test detection of tetracycline, various amounts of medicinal drugs were measured and combined with known amounts of tetracycline (see Figure 8). The following drugs were evaluated and did not create interfering background reactions: sulfanilamide (sulfa drugs), aspirin, caffeine, and quinine sulfate.

This assay was successfully applied with a cotton swap collection of a tetracycline contaminated work space. The spot test detected the presence of tetracycline dust from all collected samples. This demonstrates the efficacy of application to collection and detection of antibiotic in environmental samples. All test samples, whether solid or liquid that were examined by these procedures were able to detect the presence of tetracycline to within one percent of the the expected value. These protocols appear to work consistently.

Figure 8 demonstrates the efficacy of the spot test procedure for environmental analysis by swabbing with a cotton tipped applicator stick (see Experimental) over a contaminated surface area. A dust sampling is gathered then, which will indicate the presence of tetracycline if present (Figure 8). In addition, combinations of tetracycline with other medicinal agents such as sulfanilamide (sulfa drug derivative), aspirin, caffeine, and quinine sulfate (used for malaria treatment) gathered in a toxicology clini-

Spot Tests Results for Samples



A B C D E F

Figure 8: This figure demonstrates the results observed when applying the spot test protocol to collected samples obtained is indicated Sample A) Negative control, 15 μg of benzoic acid; Sample B) Positive control, 30 μg of salicylic acid; Sample C) Residue showing positive presence of tetracycline from dust sampling gathered by swabbing with cotton applicator stick as described in the Experimental section; Sample D) the positive indication of tetracycline from an experimental mixture of 20 μg tetracycline and an equal amount of 20 μg of sulfanilamide; Sample E) Positive indication of tetracycline from an experimental sample of tetracycline (10 μg) combined with equal amounts of aspirin (10 μg) and caffeine (10 μg); Sample F) Positive indica- tion of tetracycline from an experimental mixture of tetracycline (10 μg) and quinine sulfate (10 μg)

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cal setting can be assayed for tetracycline presence effectively (Figure 8).

TABLE 2 shows the results of multiple test sample analysis utilizing the standard curve shown in figure 5. The correct values of the test samples are shown with the experimental absorbances and results from the standard curve. Correlation of test samples to references is 0.9994, indicating extremely high similarity. A Passing-Bablok plot of reference values to test samples produced a straight line having Pearson correlation of r = 1.000. This shows that test samples are very consistent to the standard curve produced for the assay. High percent recovery also indicates efficient detection (mean = 99.25%, standard deviation = 1.988%). Colorimetric detection has advantages compared to other methods such as HPLC instrumentation which has resulted in lesser sensitivity for tetracycline[24]. A spectrophotometric method utilizing molybdenum for complexing tetracycline achieved similar sensitivity of 0.5 to 200 micrograms[25], also by differential spectrophotometry[26]. Tetracycline has been shown to be separable from a complex matrix by normal-phase thin layer chromatography[27]. Detection by capillary zone electrophoresis instrumentation is comparable, but more expensive[23].

TABLE 2: Test results for experimental determination of tetracycline utilizing a standard curve

Experimental		Actual	
Absorbance	micrograms/ mL	micrograms/ mL	Percent recovery
0.370	33.9	35.4	95.8
0.402	37.4	38.3	97.6
0.529	51.1	50.3	101.6
0.545	52.9	51.7	102.3
0.795	79.9	81.7	97.8
0.917	93.1	91.6	101.6
0.933	94.7	96.8	97.8
0.951	95.2	97.3	97.8
1.081	110.3	112.9	97.7
1.221	126.1	124.3	101.4
1.445	150.2	152.0	98.8
1.592	166.2	168.1	98.9
1.612	168.3	169.5	99.3
1.660	173.5	171.7	101.0

CONCLUSIONS

Rapid, simple, and sensitive test protocols to determine the antibiotic tetracycline are presented. The iron(III) complex formed with tetracycline is an easily recognized red-brown color, which is stable for greater than 48 hours. A strong absorption peak at 435 nm wavelength was used for assay purposes. The complex forms upon the assimilation of the monohydric phenolic group of tetracycline and does not occur for other antibiotics such as ampicillin, streptomycin, and erythromycin. Medicinal compounds which do not interfere with the assay include aspirin, sulfanilamide, benzenesulfonamide (sulfa drugs), and quinine. A rapid spot test detected as little as 30 micrograms tetracycline. An interpolative micro assay allows detection of tetracycline by visual comparison for as little as 30 micrograms. A microassay procedure, allows a detection capability for tetracycline to as little as 6 micrograms.

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