Equivalency of Ammonia (Ammonium) Spectroquant® Test Kits: Ammonia (Ammonium) by Indophenol Reaction and Photometry

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Definitions

1. Ammonia Stock Standard Solution: A concentrated solution containing method analyte prepared in the laboratory using assayed reference materials or purchased from a reputable commercial source.
2. Calibration Blank: A volume of reagent water acidified with the same acid matrix as in the calibration standards. The calibration blank is a zero standard and is used to calibrate the ammonia analyzer
3. Calibration Standard: A solution prepared from the dilution of stock standard solutions. These solutions are used to calibrate the instrument response with respect to analyte concentration.
4. Detection Limit (DL), also called Method Detection Limit (MDL) -: The minimum concentration of an analyte that can be identified, measured, and reported with 99% confidence that the analyte concentration is greater than zero.
5. Dynamic Range (DR): The concentration range over which the instrument response to an analyte is first order linear or second order quadratic.
6. Instrument Performance Check (IPC) Solution: A solution of method analyte, used to evaluate the performance of the instrument system with respect to a defined set of method criteria.
7. Laboratory Fortified Blank (LFB) - An aliquot of reagent water or other blank matrix to which known quantities of the method analytes and all the preservation compounds are added. The LFB is processed and analyzed exactly like a sample, and its purpose is to determine whether the methodology is in control, and whether the laboratory is capable of making accurate and precise measurements.
8. Laboratory Fortified Sample Matrix/Duplicate (LFM/LFMD) also called Matrix Spike/Matrix Spike Duplicate (MS/MSD): An aliquot of an environmental sample to which known quantities of ammonia is added in the laboratory. The LFM is analyzed exactly like a sample, and its purpose is to determine whether the sample matrix contributes bias to the analytical results. The background concentrations of the analytes in the sample matrix must be determined in a separate aliquot and the measured values in the LFM corrected for background concentrations.
9. Laboratory Reagent Blank (LRB) - A volume of reagent water or other blank matrix that is processed exactly as a sample including exposure to all glassware, equipment, solvents and reagents, sample preservatives, surrogates and internal standards that are used in the extraction and analysis batches. The LRB is used to determine if the method analytes or other interferences are present in the laboratory environment, the reagents, or the apparatus.
10. Minimum Reporting Level (MRL) - The minimum concentration that can be reported by a laboratory as a quantitated value for a method analyte in a sample following analysis. This concentration must not be any lower than the concentration of the lowest calibration standard for that instrument.

11. Water Sample: For the purpose of this method, a sample taken from one of the following sources: drinking water, surface water, groundwater , storm runoff, industrial or domestic wastewater.

Introduction

The method flexibility allowed in the EPA rules 40 CFR part 136.6 [1] lay out the requirements a modified analytical method must meet to be considered equivalent to a promulgated analytical method. These requirements are explained in detail in a memo authored by Richard Redding [2]:

The March 12th Methods Update Rule promulgated 136.6 which allows the regulated community more flexibility that includes:

1. Changes in equipment operating parameters such as minor changes in the monitoring wavelength of a colorimeter
2. Adjusting sample sizes or changing extraction solvents to optimize method performance in meeting regulatory requirements
3. Minor changes in reagents used where the underlying reaction and principles remain virtually the same:
   a. Changes in complexing reagent provided that the change does not produce interferences. The ammonia paper cited in section A provides an example of using a different complex reagent (citrate) other than either reagent specified in the EPA method (sodium potassium tartrate and EDTA) because it was found to be more effective and not interfere.
   b. Changes in reactants provided that the change does not produce interference. The ammonia paper cited in section A gives an example and references other examples of changing the precursor to a final product that still results in the same reaction (Berthelot reaction and formation of indophenol).

This method equivalency report will directly compare the Spectroquant® ammonium-ammonia test kits with the simple method modifications listed in Richard Reading's memo:

1. Change the phenol reactant from phenol to substituted phenols 2-chlorophenol or thymol (3(b) above)
2. Change the complexing reagent to address interferences. (3(a) above. See Table 5 below for complexing reagent changes.
3. Change chlorine source from hypochlorite to dichloroisocyanuric acid. (3(b) above)
4. Change the monitoring wavelength to a higher wavelength (Bathochromic shift) to meet the new maxima for the substituted phenols. (1 above)
5. Changes in sample size with test kit vial to meet the new Dynamic Range. (2 above)

Indophenol (Berthelot) Reaction

Chemical Reactions and Spectroscopy

Ammonia-Ammonium Chemistry

The Spectroquant® test kits and all of the Spectroquant® Quality Control (QC) samples identified the active ingredient used in their analyses as ammonium (NH_4^+). The EPA approved methods that utilize spectroscopic determination the same compound identify the active ingredient used in their analyses as ammonia (NH_3). These compounds are in equilibrium in an aqueous solution with the ammonium prevalent at low pH and the ammonia prevalent at high pH.

$$\underset{\text{Low pH}}{NH_4^+} \rightleftarrows \underset{\text{High pH}}{NH_3}$$

Figure 1: Ammonium-Ammonia Equilibrium

This equilibrium is used to:

1. Produce the ammonium salts that are stable at room temperature and can be used to prepare standard solutions:
    a. Ammonium Chloride (NH_4Cl)
    b. Ammonium Sulfate ($(NH_4)_2SO_4$)
2. Convert ammonium to ammonia:
    a. Raise the pH to 9.5 for distillation of ammonia 4500-NH_3 B-1997 [3]
    b. Convert ammonium to ammonia for the Berthelot reaction pH (9.5-11) [4].
3. Convert ammonia to ammonium:
    a. Trap distilled ammonia from the distillation step with 0.04 *N* H_2SO_4 for the Berthelot reaction 4500-NH_3 B-1997 [3] .
    b. Preserve aqueous samples , $pH < 2$ [1].

As such, once the concentration of the final analyte has been adjusted to the higher pH, it produces the ammonia needed to react with chlorine to produce mono-chloramine required for the Berthelot reaction. The final concentration analytical results reported for the compound of interest, ammonium or ammonia, are the same. (i.e. mg/L NH_3-N = mg/L NH_4-N).

Analytical Standards

For this study, all analytical standards were prepared as a NH_3-N or NH_4-N. The ammonia standard was prepared from NH_4Cl (Sigma A9434, Assay ≥ 99.5) which was dried overnight at 104 ºC and stored in a desiccator until cool. This ammonium chloride was then used within 8 hours of cooling to prepare the:

1. Standard Stock Solution 1 (1000 mg/L NH_3-N, 1216 mg/L NH_3, 1000 mg/L NH_4-N,, 1288 mg/L NH_4^+):
    a. 3.819 g (± 0.0001 g) of the dried ammonium chloride was dissolved in ammonia free water in a Class A 1000 mL volumetric flask.
    b. Ammonia free water was added to the mark (1000 mL).
    c. Stock Solution 1 was mixed completely by flask inversion.
2. Standard Stock Solution 2 (100.00 mg/L NH_3-N, 121.6 mg/L NH_3, 100.00 mg/L NH_4-N,, 128.8 mg/L NH_4^+):
    a. 100 mL of Standard Stock Solution 1 was transferred with a Class A 50 mL pipet to a Class A 1000 mL volumetric flask.
    b. Ammonia free water was added to the mark (1000 mL).
    c. Stock Solution 2 was mixed completely by flask inversion.
3. Standard Stock Solution 3 (10.00 mg/L NH_3-N, 12.16 mg/L NH_3, 10.00 mg/L NH_4-N,, 12.88 mg/L NH_4^+):
    a. 100 mL of Standard Stock Solution 2 was transferred with a Class A 50 mL pipet to a Class A 1000 mL volumetric flask.
    b. Ammonia free water was added to the mark (1000 mL).
    c. Stock Solution 3 was mixed completely by flask inversion.

All standards and spike solutions were prepared as per Table 1 below.

Table 1: Standard Preparation

Standard (mg/L NH_3-N or mg/L NH_4-N)	**Standard Stock Solution 1 (mL) (Diluted to 100 mL)**
150	15
120	12
Standard (mg/L NH_3-N or mg/L NH_4-N)	**Standard Stock Solution 2 (mL) (Diluted to 100 mL)**
100	Use as is.
80	80
60	60
40	40
20	20

Table 1: Standard Preparation	
15	15
Standard (mg/L NH_3-N or mg/L NH_4-N)	**Standard Stock Solution 3 (mL) (Diluted to 100 mL)**
10	Use as is
8	80
4	40
3	30
2	20
1	10
0.5	5
0.1	1
0.05	*Note: 1 ml diluted to 200 mL*

Distillation or Other Sample Preparation

The samples used in this method equivalency study were either standards prepared in ammonia free water or wastewater effluent that was filtered through a 0.45 µm Whatman PES syringe filter to remove turbidity.

The Standard Methods for the Examination of Water and Wastewater [3] methods approved by EPA [1] allows the analyses of these ammonia samples without distillation. Filtration is identified in the Spectroquant® product flyers and is acceptable under the EPA approved method 4500-NH_3 F (1)-1997.

1. 4500-NH_3 A (1)-1997 "Methods D, E, F, G, and H may be used either with or without sample distillation."
2. 4500-NH_3 F (1)-1997 "Remove interfering turbidity by distillation or filtration".

Classical Phenol Reaction

The Indophenol or Berthelot reaction has been utilized for the detection of ammonia in aqueous samples with in-depth studies and reviews published [4-6]. The overall reaction proposed as a 3-step process[6] in which:

1. The ammonia reacts with a chlorine source (hypochlorite or sodium dichloroisocyanurate) in a high pH solution to produce monochloroamine.

2. The monochloroamine then react with a phenol group to produce benzoquinone monochloroamine intermediate.
3. The benzoquinone monochloroamine intermediate then reacts with a second phenol to produce indophenol dye.

Other researches have proposed different initial and intermediate steps [4, 5], but the final indophenol product is recognized by all authors.

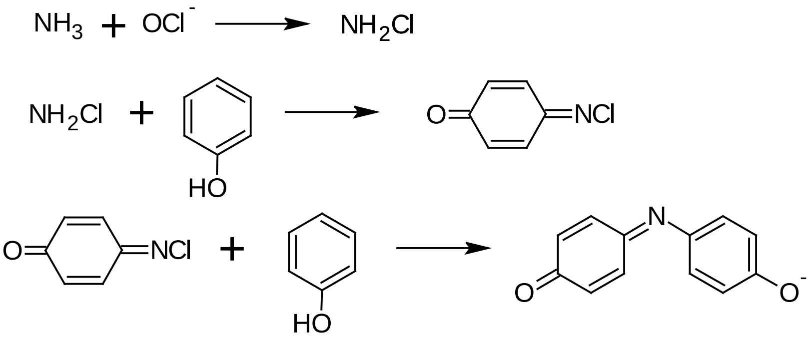

Figure 2: Classical Phenol Berthelot Reaction

Sodium Nitroferricyanide

The addition of sodium nitroprusside or sodium nitroferricyanide to the reaction has been identified as either a catalyst or a reagent. The exact function of this compound has not been determined, but its addition increases the rate of reaction. The exact compound that acts as a catalyst or reagent has been studied [6] and an intermediate equilibrium between sodium nitroferricyanide and sodium aquopentacyanoferrate is suggested.

$$Na_2\left[Fe\left(CN\right)_5 NO\right] \rightleftarrows Na_3\left[Fe\left(CN\right)_5 H_2O\right]$$

Figure 3: Sodium Nitroferricyanide Equilibrium

Spectrophotometric analyses of a sodium nitroprusside solution at a pH of 7 and a pH of 11 shows the growth of a peak 399 nm which could be either the sodium nitroprusside or sodium aquopentacyanoferrate compound.

But whatever the chemical compound present in solution, the reaction yields the yellow color seen in the method blanks no matter what phenol compound is used.

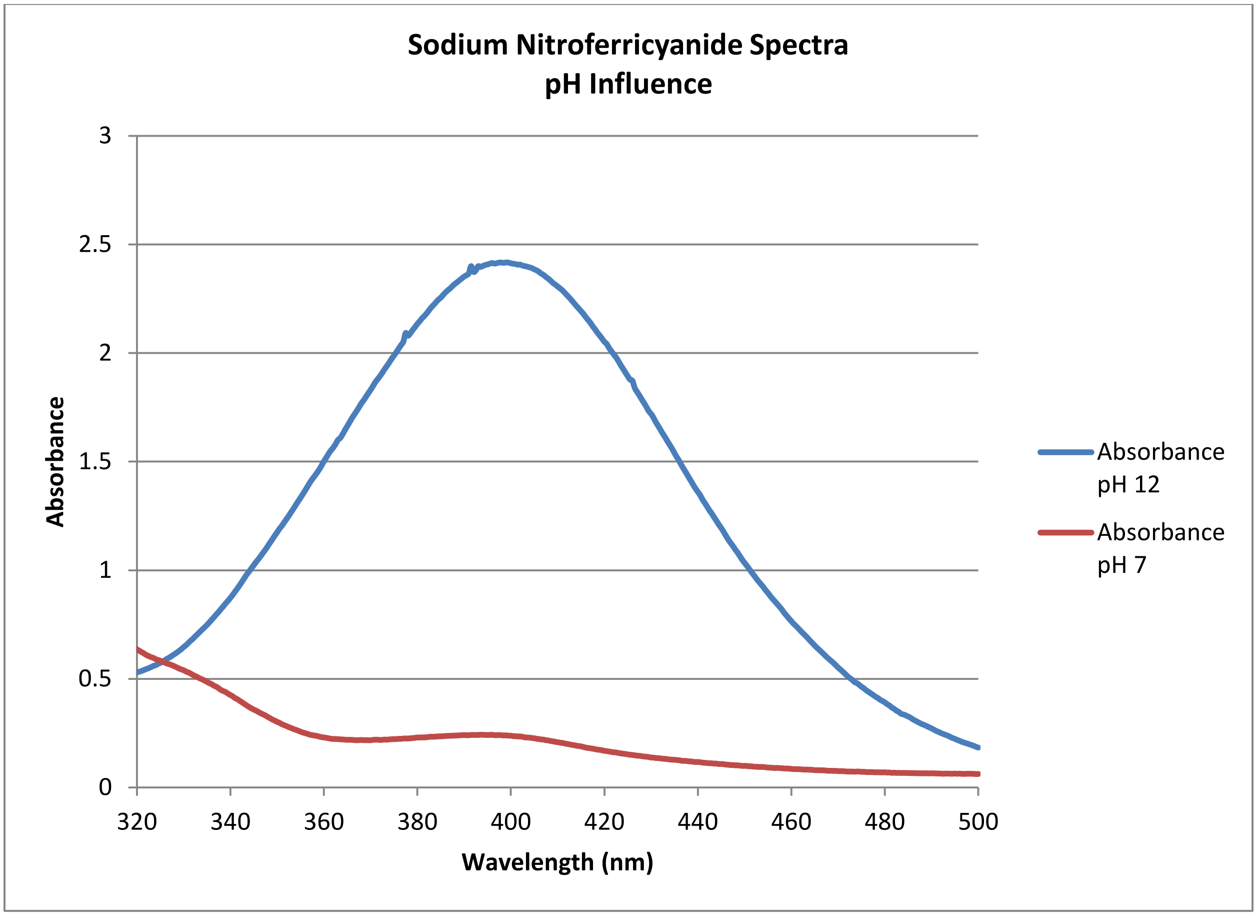

Figure 4: pH Influence on Sodium Nitroferricyanide

Substituted Phenols

There have been many substitutes phenols used in the Berthelot's reaction for the detection of ammonia. In many cases, the substituted phenol used was selected for its reactivity or lower toxicity. In all cases, the final indophenol dye forms contain the two aromatic phenyl rings with the substituted ring sites intact. Some of the substituted phenols are listed in Table 2 below.

Table 2: Phenols and Substituted Phenols Used in the Berthelot's Reaction	
Analytical Method	**Phenol or Substituted Phenol**
Standard Methods 4500-NH_3-F,G,H 1997 [3]	Phenol
USGS I-4523-85 [7]	Phenol
Patton [6]	Phenol or 2 Chlorophenol *Note: Other substituted phenols reported, but experimental directions were for 2-Chlorophenol.*
Krom [5]	Phenol or Sodium Salicylate
Searle [4]	***Review Article:*** *Phenol, Sodium Salicylate Other substituted phenols reported.*
EPA 350.1 [8]	Phenol or Sodium Salicylate
ISO 7150/1 [9]	Sodium Salicylate
Spectroquant® Test Kits	
1.14739	2 Chlorophenol
1.14752	Thymol
1.14558	2 Chlorophenol
1.14544	2 Chlorophenol
1.00683	2 Chlorophenol
1.14559	2 Chlorophenol

The substituted phenols reactions are detailed in Figures 4-5 below. They all produce an indophenol final product.

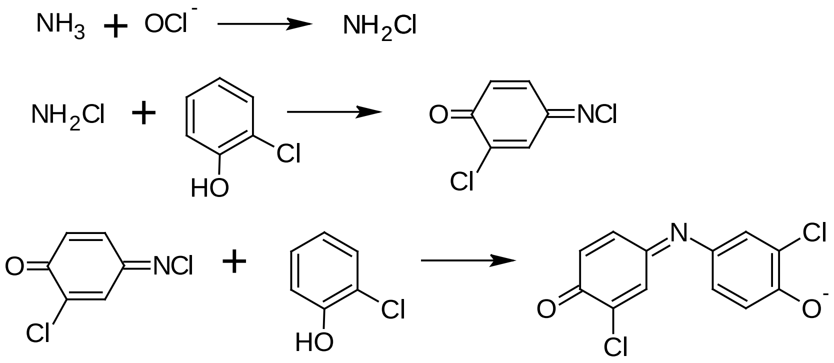

Figure 5: 2-Chlorophenol Berthelot Reaction

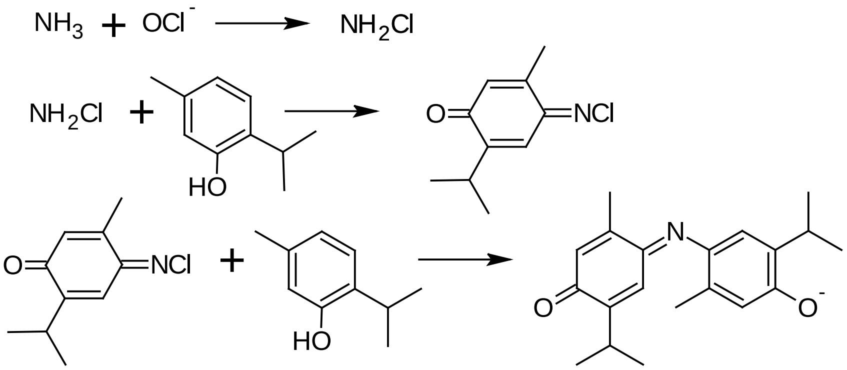

Figure 6: Thymol Berthelot Reaction

Reaction Rate

The reaction rate for the substituted phenols in the Spectroquant® test kits at room temperature are four to six times faster than the classical phenate method Standard Methods 4500-NH_3 (F)-1997. The Spectroquant® test kits provided a quantitative result within 15 minutes for the 2-chlorophenol and 10 minutes for the Thymol in the open in the laboratory. The classical phenate method had to react 60 minutes in the dark before a quantitative result could be obtained.

Chlorine Sources

The source of chlorine in the Berthelot reaction has reported to be key for the following areas [4]:

1. The chlorine source must be stable so as to produce reliable quantitative sources.
2. The chlorine source must react with the ammonia at room temperature so as to produce quantitative results.
3. The chlorine source must be easily and safely handled by laboratory analysts.

The 3 major chlorine reagents utilized in the Berthelot reaction are summarized in Table 3 below.

Table 3: Chlorine Sources for the Berthelot Reaction			
Chlorine Reagent	**Stabile at Room Temperature**	**React at Room Temperature**	**Easily and Safely Handled**
Hypochlorite (Bleach)	Short Term	Yes	Yes
Sodium Dichloroisocyanurate or Dichloroisocyanuric Acid	Yes	Yes	Yes
Chloramine-T	Yes	No	Yes

Indophenol Spectra

The absorption spectra of the final indophenol product are impacted by both the pH of the final solution and the interaction of the substituted compounds on the phenyl ring. The classical phenol reaction produces an absorbance maximum (maxima) in the ranges listed in Table 4 below:

Table 4: Phenols Instrument Maximum (Maxima) Used in the Berthelot's Reaction	
Analytical Method	**Classical Phenol Absorbance Maximum (Maxima) nm**
Standard Methods 4500-NH_3-F,G,H 1997 [3]	F. 640 G. 630-660 H. 630
USGS I-4523-85 [7]	630
Patton [6]	635-637
Krom [5]	630
EPA 350.1 [8]	660***** **** Note:*** *Method calls for phenol in reagent preparation, but the manifold figure lists salicylate reagent.*

The variation of the absorbance maxima indicates that there is a broad peak maximum that allows quantitation of the indophenol product over a large wavelength range. This can be seen in Figure 7 below.

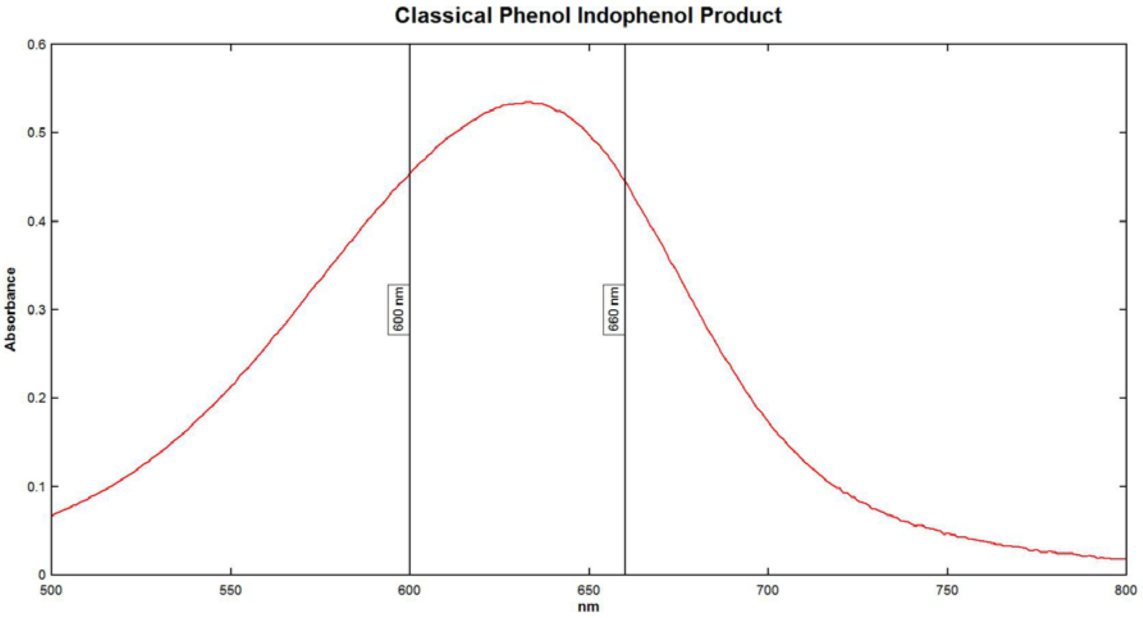

Figure 7: Classical Phenol Indophenol Spectra

The influence of the additional compounds on the phenyl ring in the indophenol compound along with the final solution pH can cause a hypsochromic (lower wavelength) or bathochromic (higher wavelength) shift of the maxima. The bathochromic shift due to the additional compounds on the phenyl ring can be attributed either the charge delocalization of the phenyl ring due to the electron withdrawing chlorine atom in the 2-Chlorophenol or the steric strain due to the 2-propyl and methyl compounds in the thymol as illustrated in Figure 8 below.

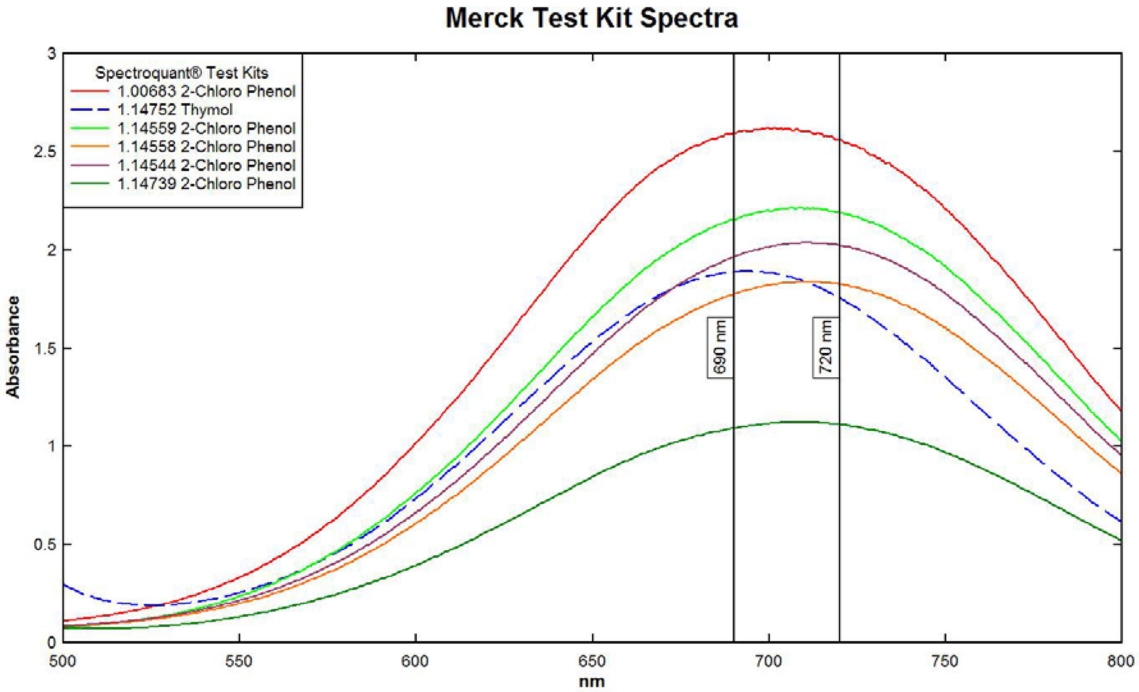

Figure 8: Spectroquant® Test Kits Absorbance Spectra

In all cases with the substituted phenols, a maxima band can be identified and accurate quantitation of the indophenol dye or the ammonia present can be determined.

Maximum Wavelength

The broad band indophenol maxima seen for each Spectroquant ®test kit had a slight variation on the absolute maximum used for each test kit. To determine the absolute maximum for each test kit, a standard for ammonia whose concentration was at or near the maximum concentration listed in the test kit product sheet was analyzed. These maximum wavelengths are summarized in Table 5 below. An additional wavelength of 690 nm was used to take into account Spectroquant® fixed filter colorimeters.

Table 5 Spectroquant® Test Kit Wavelengths

1.00683 High Range Check Samples	690 nm
	710 nm
1.00683 Low Range Check Samples	690 nm
	703 nm
1.14739 Check Samples	690 nm
	708 nm
1.14559 Check Samples	690 nm
	710 nm
1.14544 Check Samples	690 nm
	711 nm
1.14558 Check Samples	690 nm
	710 nm
1.14752 Check Samples	690 nm

Calibration and Linear Correlation Coefficient

Utilizing both the absorbance maximum determined experimentally for the Spectroquant® test kit and 690 nm, the Thermo Scientific Genesys 10S was calibrated with a minimum of six (6) standards and a linear correlation coefficient was determined. The standards bracketed the Spectroquant® test kit concentrations listed in the product flyer.

The correlation coefficient was >0.999 in all cases indicating that the Spectroquant® test kits can produce a linear calibration. The calibration standards using mg/L (NH_3-N or NH_4-N) for each test kit along with the linear Correlation Coefficients are summarized in Table 6 below.

The calibration values for the traditional phenate Berthelot reaction Standard Methods 4500-NH_3 (F) [3] produced a linear calibration line and a correlation coefficient > 0.999 as summarized in Table 7 below. Therefore, the substituted phenols used in the Spectroquant ® test kit performed in an equivalent manner as the classical phenate method.

Table 6: Spectroquant® Test Kit Calibration Standards and Correlation Coefficient

Test Kit	Wavelength	Standard 1*	Standard 2*	Standard 3*	Standard 4*	Standard 5*	Standard 6*	Standard 7*	Standard 8*	Standard 9*	Correlation Coefficient
1.00683 High	**690 nm**	0.000	8.000	10.000	20.000	40.000	80.000	100.000	120.000	150.000	0.99972
	710 nm	0.000	8.000	10.000	20.000	40.000	80.000	100.000	120.000	150.000	0.99973
1.00683 Low	**690 nm**	0.000	2.000	4.000	8.000	10.000	20.000	40.000	60.000		0.99993
	703 nm	0.000	2.000	4.000	8.000	10.000	20.000	40.000	60.000		0.99994
1.14739	**690 nm**	0.000	0.050	0.100	0.500	0.700	1.000	2.000			0.99995
	708 nm	0.000	0.050	0.100	0.500	0.700	1.000	2.000			0.99996
1.14559	**690 nm**	0.000	4.000	8.000	10.000	20.000	40.000	60.000	80.000		0.99989
	710 nm	0.000	4.000	8.000	10.000	20.000	40.000	60.000	80.000		0.99991
1.14544	**690 nm**	0.000	0.500	1.000	2.000	4.000	8.000	10.000	15.000		0.99973
	711 nm	0.000	0.500	1.000	2.000	4.000	8.000	10.000	15.000		0.99973
1.14558	**690 nm**	0.000	0.500	1.000	2.000	4.000	8.000				0.99982
	710 nm	0.000	0.500	1.000	2.000	4.000	8.000				0.99975
1.14752	**690 nm**	0.000	0.100	0.500	1.000	2.000	3.000				0.99992

* Standard Concentration NH_3-N or NH_4-N mg/L

Table 7: Standard Methods 4500-NH_3 (F) Phenate Calibration Standards and Correlation Coefficient

Test Kit	Wavelength	Standard 1*	Standard 2*	Standard 3*	Standard 4*	Standard 5*	Standard 6*	Standard 7*	Standard 8*	Correlation Coefficient
Phenate	**633 nm**	0.000	0.050	0.100	0.500	0.700	1.000	2.000	120.000	0.99986

* Standard Concentration NH_3-N or NH_4-N mg/L

Accuracy, Percent Recovery and Precision

Accuracy (Spectroquant® QC Sample-LFB)

Quality control check samples were obtained from Spectroquant® (Table 8) and used in the equivalency evaluation of the Spectroquant® test kits ability to recover free ammonia (ammonium). The control check concentration (accuracy) and precision are summarized in Tables 9-10 below. The percent recoveries for each Spectroquant® test kit and Standard Methods 4500-NH_3 (F) [3] for the quality control check samples used are summarized in Table 9.

$$\left(\frac{\text{Experimental Value}}{\text{Expected Value}}\right)*100 = \text{Percent Recovery LFB}$$

Experimental Value = LFB Concentration determined experimentally

Expected Value = Known LFB concentration

Figure 9: LFB Percent Recovery

Precision (Spectroquant® QC Sample-LFB)

Precision is defined in Standard Methods for the Examination of Water and Wastewater, Part 1000 and in EPA guidance documents as either the confidence interval about the mean or the Percent Relative Standard Deviation (%RSD).[10-12]

$$\text{Percent Relative Standard Deviation} = \left(\frac{s}{\bar{X}}\right)\times 100$$

Figure 10: Percent Relative Standard Deviation

Table 8: Spectroquant® Quality Control Check Samples (LFB-IPC)

Spectroquant® Check Sample	Label Concentration (NH_4-N mg/L)	Check Sample Actual Concentration (NH_4-N mg/L)	Check Sample Precision (± mg/L)
1.25022.0100	0.400	0.396	0.012
1.25023.0100	1.00	0.98	0.04
1.25024.0100	2.00	2.03	0.07
1.25025.0100	6.00	5.98	0.13
1.25026.0100	12.0	12.0	0.4
1.25027.0100	50.0	51.0	1.2

The %RSD was calculated for the Spectroquant® and Standard Methods 4500-NH_3 (F) [3] check samples that were used for the accuracy evaluation. The number of replicate concentration determinations at the maximum wavelength determined for each Spectroquant® test kit and at

690 nm was five (5) replicates. The mean and standard deviation was then calculated and these values were used to determine %RSD. The %RSD for reach quality controls sample is summarized in Table 10 below.

Some of the experimental data had a standard deviation of zero (0). In these cases, the %RSD was set to 0.00%. In other cases the standard deviation was of such a low order of magnitude, that the calculated %RSD was < 0.0049 % and the value was rounded to 0.00%.

The accuracy or recovery of the Spectroquant® check samples by either the Spectroquant® test kits or the classical phenate method was no lower than 96.77% and no higher than 110.61%. The %RSD of the Spectroquant® check samples by either the Spectroquant® test kits or the classical phenate method was no greater than 0.12%.

These efficient recoveries of 3rd party check samples and the low %RSD shows that the Spectroquant® will produce equivalent accurate and precise results when compared to Standard Methods 4500-NH_3 (F) [3]. This is emphasized in a direct comparison between a Spectroquant® test kit and the classical phenate method with a calibration range that brackets two Spectroquant® Check sample, Tables A-B below.

Table A: LFB Recovery Comparison Classical Phenate - Spectroquant®

Spectroquant® Check Sample	**Label Concentration (NH_4-N mg/L)**	**Classical Phenate**	**1.14739**	
		633 nm	**690 nm**	**708 nm**
1.25022	0.4	100.51%	107.32%	107.07%
1.25023	1	107.55%	110.61%	102.45%

Table B: Classical Phenate %RSD Comparison Classical Phenate - Spectroquant®

Spectroquant® Check Sample	**Label Concentration (mg/L)**	**Classical Phenate**	**1.14739**	
		633 nm	**690 nm**	**708 nm**
1.25022	0.4	0.00%	107.32%	107.07%
1.25023	1	0.00%	110.61%	102.45%

Laboratory Reagent Blank (LRB)

A volume of reagent water or other blank matrix was processed exactly as a sample including exposure to all glassware, equipment, solvents and reagents, sample preservatives, that are used in the analysis. The LRB was run five times for each Spectroquant® test and the average is reported in Table 11 below. In all cases, ammonia was not seen at a level above 0.00 mg/L NH_3-N or NH_4-N

Table 9: Spectroquant® and Classical Phenate Quality Control Accuracy (LFB-IPC NH_3-N or NH_4-N % Recovery)

Label Concentration (mg/L)	1.00683 High Range Check Samples		1.00683 Low Range Check Samples		1.14739 Check Samples		1.14559 Check Samples		1.14544 Check Samples		1.14558 Check Samples		1.14752 Check Samples	Classical Phenate
	690 nm	**710 nm**	**690 nm**	**703 nm**	**690 nm**	**708 nm**	**690 nm**	**710 nm**	**690 nm**	**711 nm**	**690 nm**	**710 nm**	**690 nm**	**633 nm**
0.4					107.32%	107.07%								100.51%
1					110.61%	102.45%					105.20%	104.69%	105.71%	107.55%
2									101.13%	101.92%	100.94%	100.49%	100.00%	
6	100.89%	101.61%	97.06%	101.61%			98.93%	99.88%	97.53%	98.11%	99.33%	99.18%		
12	102.86%	103.49%	99.12%	99.86%			100.89%	101.13%	97.37%	97.28%				
50	97.80%	98.01%	99.63%	99.54%			96.96%	96.77%						

Table 10: Spectroquant® and Classical Phenate Quality Control Precision (LFB % RSD)

Label Concentration (mg/L)	1.00683 High Range Check Samples		1.00683 Low Range Check Samples		1.14739 Check Samples		1.14559 Check Samples		1.14544 Check Samples		1.14558 Check Samples		1.14752 Check Samples	Classical Phenate
	690 nm	710 nm	690 nm	703 nm	690 nm	708 nm	690 nm	710 nm	690 nm	711 nm	690 nm	710 nm	690 nm	633 nm
0.4					0.00%	0.00%								0.00%
1					0.00%	0.00%					0.05%	0.05%	0.00%	0.00%
2									0.05%	0.05%	0.12%	0.02%	0.00%	
6	0.07%	0.07%	0.03%	0.03%			0.05%	0.05%	0.12%	0.02%	0.01%	0.02%		
12	0.02%	0.04%	0.02%	0.03%			0.02%	0.02%	0.01%	0.02%				
50	0.00%	0.01%	0.02%	0.03%			0.01%	0.01%						

Table 11: Spectroquant® Laboratory Reagent Blank (mg/L NH_3-N or NH_4-N))

1.00683 High		1.00683 Low		1.14739		1.14559		1.14544		1.14558 s		1.14752
690 nm	710 nm	690 nm	703 nm	690 nm	708 nm	690 nm	710 nm	690 nm	711 nm	690 nm	710 nm	690 nm
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Matrix Spikes

A wastewater matrix was utilized in determining the matrix effects on the spike percent recovery, precision and the relative percent difference between the matrix spike (MS) and the duplicate spike (MSD).

A sample of wastewater effluent was obtained from a wastewater plant final clarifier prior to disinfection. This sample was then filtered through a 0.45 µm syringe filter prior to matrix analyses.

The wastewater plant laboratory staff indicated that the ammonia analyses performed in their laboratory consistently had results of ammonia concentrations of < 0.5 mg/L NH3-N. The Spectroquant® test kit 14739 was selected for the test as it has the lowest reporting limit. Matrix spike concentration was selected to be at the midpoint of the Spectroquant® test kit range (1 mg/L NH_3-N). Each spectroscopic analysis was measured five 5) times and the average is reports and the standard deviation was used in the % RSD calculation.

Figures 11-12 provide the calculations used. The percent recovery, RPD and %RSD for the samples are summarized in Table 12-13 below.

$$\left(\frac{Spiked\ Value - (s\ x Unspiked\ Value)}{Concentration\ of\ Spike}\right) * 100 = \text{ Percent Recovery LFM}$$

$$Spiked\ Value = \text{LFM concentration determined experimentally}$$
$$Unspiked\ Value = \text{Concentration of sample before spiking}$$
$$s = Dilution\ Correction$$

Figure 11: Percent Recovery LFM

$$\left(\frac{(LFM - LFMD)}{\left(\frac{LFM + LFMD}{2}\right)}\right) * 100 = \text{RPD}$$

LFM = Concentration determined for LFM

$LFMD$ = Concentration determined for LFM duplicate

Figure 12: Relative Percent Difference (RPD)

The results show that the Spectroquant® test kit can determine ammonia in a wastewater matrix.

Table 12: Matrix Spike Percent Recovery and RPD		
	690 nm	**708 nm**
Final Effluent (NH_3-N or NH_4-N mg/L)	0.165	0.174
Matrix Spike (NH_3-N or NH_4-N mg/L)	1.150	1.147
Matrix Spike Percent Recovery	98.50%	97.30%
Matrix Spike Duplicate (NH_3-N or NH_4-N mg/L)	1.158	1.153
Matrix Spike Duplicate Percent Recovery	99.30%	97.90%
Relative Percent Difference	0.69%	0.52%

Table 13: Matrix Spike and Spike Duplicate %RSD		
	690 nm	**708 nm**
Final Effluent	0.00%	0.00%
Matrix Spike	0.00%	0.00%
Matrix Spike Duplicate	0.00%	0.00%

Method Detection Limits

The Method Detection Limit (MDL) was calculated for each Spectroquant® test kit following the requirements in Appendix B, 40 CFR part 136 [13]. Each Spectroquant® test kit product flyer and test kit contained an ammonia (ammonium) range for which the test kit reagents were at a concentration to produce complete reaction of the ammonia to form in indophenol. The concentration of sample utilized for each Spectroquant® test kit MDL was determined to be 2-5 times the concentration of the lowest concentration listed on the Spectroquant® test kit or product flyer.

The results for the Spectroquant® test kit MDL are at or below the MRL listed on the Spectroquant® product flyer (Table 14). These MRL vs. MDL results for the Spectroquant® test kits concludes that the Spectroquant® test kits with the substituted phenols can provide an indophenol chromophore that responds like the classical phenate method and that the dynamic range listed on each Spectroquant® product flyer is accurate.

Table 14: Method Reporting Limit - Method Detection Limits			
Spectroquant® Test Kit	**Wavelength**	***MRL* *Lowest Concentration Listed for Spectroquant® Test Kit* *(NH_3-N or NH_4-N mg/L)***	**MDL (NH_3-N or NH_4-N mg/L)**
1.00683 High Range	**690 nm**	***5.00***	0.438
	710 nm	***5.00***	0.331
1.00683 Low Range	**690 nm**	***2.00***	0.257
	703 nm	***2.00***	0.321
1.14739	**690 nm**	***0.010***	0.010
	708 nm	***0.010***	0.008
1.14559	**690 nm**	***4.00***	0.285
	710 nm	***4.00***	0.295
1.14544	**690 nm**	***0.50***	0.066
	711 nm	***0.50***	0.057
1.14558	**690 nm**	***0.20***	0.061
	710 nm	***0.20***	0.061
1.14752	**690 nm**	***0.05***	0.031

Berthelot-Indophenol Method Reagent Comparison

An overall review of the ammonia spectrophotometric analytical reagents and their uses is summarized in Table 15 below. In the Introduction section in this report, the conditions that are approved under the currently promulgated 40 CFR part 136.6 were expanded on in detail in the Richard Reding memo [2]:

1. Minor changes in reagents used where the underlying reaction and principles remain virtually the same:
    a. Changes in complexing reagent provided that the change does not produce interferences. The ammonia paper cited in section A provides an example of using a different complex reagent (citrate) other than either reagent specified in the EPA method (sodium potassium tartrate and EDTA) because it was found to be more effective and not interfere.
    b. Changes in reactants provided that the change does not produce interference. The ammonia paper cited in section A gives an example and references other examples of changing the precursor to a final product that still results in the same reaction (Berthelot reaction and formation of indophenol).

The reagent changes when compared to one approved ammonia analysis Standard Methods 4500 NH_3 -F were:

1. Substituted phenols (2-Chlorophenol or Thymol).
2. Chlorine source was changed from hypochlorite to dichloroisocyanuric acid.
3. Chelation reagent was changed from trisodium citrate to 1 Hydroxy Ethylidene 1,1 Diphoshonic Acid
4. Sorbitol was added to the dry Spectroquant® reagent to prevent caking. It did not interfere with the Berthelot reaction.

As other EPA approved ammonia tests listed in Table 15 have changed one or all of these same reagents and the results from all of the results listed previously, these modification in the Spectroquant® test kits fall under the detailed guidelines in Reding memo and as such the Spectroquant® tests kits evaluated are equivalent to promulgated EPA ammonia indophenol methods.

Table 15: Ammonia-Ammonium Berthelot Method Reagent Comparison

Reagent	**SM 4500-NH_3-F,G,H 1997**	**USGS I-4523-85**	**Patton (et al) ***	**Krom***	**EPA 350.1**	**ISO**	**Spectroquant®**
Berthelot Phenol	Phenol	Phenol	2 Chlorophenol	Phenol or Salicylate	Phenol or Salicylate?	Salicylate	2 Chlorophenol or Thymol
pH Adjustment Reagent	Sodium Hydroxide	Sodium Hydroxide	Phosphate-Borate	Sodium Hydroxide	Sodium Hydroxide	Sodium Hydroxide	Sodium Hydroxide
Oxidizing Agent	Sodium Hypochlorite	Sodium Hypochlorite	Sodium Hypochlorite	Sodium Dichloroisocyanurate	Sodium Hypochlorite	Sodium Dichloroisocyanurate	Dichloroisocyanuric Acid
Catalyst?	Sodium Nitroprusside	Sodium Nitroprusside	Sodium Nitroprusside	Sodium Nitroprusside	Sodium Nitroprusside	Sodium Nitroprusside	Sodium Nitroprusside
Chelation Reagent	Trisodium Citrate (F) Disodium EDTA or Potassium Tartarate (G) Disodium EDTA (H)	Trisodium Citrate Potassium Tartrate		Trisodium Citrate	Disodium EDTA	Trisodium Citrate	1 Hydroxy Ethylidene 1,1 Diphoshonic Acid
Quantitation Wavelength (nm)	640 (F) 630-660 (G) 630 (H)	630	650	660 (Salicylate) 630 (Phenol)	660	655	690-715
Anticaking, Solubilizing, Stabilizing, Binding Agent							Sorbitol

**: From EPA 40 CFR part 136 Table IB footnote 60*

Summary and Conclusion

Summary

This equivalency study has provided the literature review information, EPA 40 CFR part 136.6 requirements and experimental data to support the equivalency of the current Spectroquant® ammonium test kits with the EPA current promulgated ammonia phenate methods listed in the 40 CFR part 136. The changes listed in the Spectroquant® test kit and the equivalency requirements are listed in the Table 16 below.

Conclusion

In conclusion, these changes in the Spectroquant® test kits (or future test kits with the same equivalent chemistry) produce an equivalent set of tests to the current EPA promulgated ammonia phenate methods listed in the 40 CFR part 136.

Table 16: Method Equivalency Summary	
Spectroquant® Change	**136.6 Requirement**
Substituted phenols (2-Chlorophenol or Thymol)	No change in the overall Berthelot reaction. 1. Other referenced methods in 40 CFR part 136 use substituted phenols. 2. An indophenol was formed with the substituted compounds changing the wavelength for maximum absorbance changing. 3. Accuracy, Precision, MRL and MDL from the experimental data were acceptable.
Chlorine source was changed from hypochlorite to dichloroisocyanuric acid.	No change in the overall Berthelot reaction. 1. Other referenced methods in 40 CFR part 136 use other chlorine sources Sodium Dichloroisocyanurate, which is the acid salt of Dichloroisocyanuric Acid 2. An indophenol was formed with the substituted compounds changing the wavelength for maximum absorbance changing. 3. Accuracy, Precision, MRL and MDL from the experimental data were acceptable.
Chelation reagent was changed from trisodium citrate to 1 Hydroxy Ethylidene 1,1 Diphoshonic Acid	No change in the overall Berthelot reaction. 1. Cations were chelated in the MS/MSD and did not affect Accuracy or Precision
Sorbitol was added to the dry Spectroquant® reagent to prevent caking.	No change in the overall Berthelot reaction.

References

1. EPA, *Guidelines Establishing Test Procedures for the Analysis of Pollutants Under the Clean Water Act; Analysis and Sampling Procedures*. 2012. p. 29758-29846.
2. Reding, R., *Flexibility to Modify CWA Methods*, E. Engineering & Analytical Support Branch, OST, Editor. 2007, EPA.
3. Eaton, E., Baird, R., Rice, E., , ed. *Standard Methods for the Examination of Water and Wastewater, 22nd Edition*. 22 ed. 2012, APHA, AWWA, WEF.
4. Searle, P.L., *The berthelot or indophenol reaction and its use in the analytical chemistry of nitrogen. A review.* Analyst, 1984. **109**(5): p. 549-568.
5. Krom, M.D., *Spectrophotometric determination of ammonia: a study of a modified Berthelot reaction using salicylate and dichloroisocyanurate.* Analyst, 1980. **105**(1249): p. 305-316.
6. Patton, C.J. and S.R. Crouch, *Spectrophotometric and kinetics investigation of the Berthelot reaction for the determination of ammonia.* Analytical Chemistry, 1977. **49**(3): p. 464-469.
7. USGS, *Nitrogen, ammonia, colorimetric, indophenol, automated-segmented flow*. 1985, USGS.
8. EPA, *350.1 Determination Of Ammonia Nitrogen By Semi-Automated Colorimetry. Rev 2.0*. 1993.
9. ISO, *Water Quality - Determination of ammonium -Part 1 : Manual spectrometric method*. 1984.
10. EPA, *Chapter 5: Calculation Of Precision, Rias, And Method Detection Limit For Chemical And Physical Measurements*. 1984.
11. EPA, *Protocol for the Evaluation of Alternate Test Procedures for Organic and Inorganic Analytes in Drinking Water*. 2015.
12. EPA, *Protocol for Review and Validation of New Methods for Regulated Organic and Inorganic Analytes in Wastewater Under EPA's Alternate Test Procedure Program*. 2016.
13. EPA, *Subchapter D 40 CFR part 136 Guidelines Establishing Test Preoceedures for the Analysis of Pollutants*. 2001.

Appendix

EPA Method Equivalency Check-off Table from Richard Reding Memo, *Flexibility to Modify CWA Methods*, 2007	
Equivalency Requirement	**Section in Report**
Concentrations of calibration standards. Document the range of the concentrations of material used to establish the relationship between response of the measurement system and analyte concentration.	Yes, Tables 1, 6 and 7
%RSD or correlation coefficient of calibration regression.	Yes, Tables 6, 7, B and 10
Performance range tested with units.	Yes, Tables 6, 7, 9, 10, 14, A and B.
Sample(s) used in initial demonstration have the recommended preservative, where applicable.	No preservation was needed. But, the Spectroquant® kits list adjusting the pH before performing the Berthelot reaction, which is also mentioned in Standard Methods 4500 NH3 (A 3)-1997 Ammonia was either in a standard, from a 3rd party or from a wastewater plant. The standards were used the same day they were made. The 3rd part QC standards were used as received The wastewater sample was used within 1 hour of collection.
Sample(s) used in initial demonstration met recommended holding times, where applicable.	The wastewater sample was used within 1 hour of collection.
Interferences.	None, MS/MDS Tables 11 and 12
Document the qualitative identification criteria used.	LFB percent recovery from 3rd party QC samples. Tables A and 10. %RSD from 3rd party QC samples. Tables B and 11. MDL determination and comparison to MRL of Spectroquant® test kits. Table 13. MS/MSD . Tables 11 and 12
Performance evaluation studies performed for analytes of interest, where available.	See Tables 2, 4, and 14. References 4-6
Latest study sponsor or title	NA
Latest study number.	NA

EPA Method Equivalency Check-off Table from Richard Reding Memo, *Flexibility to Modify CWA Methods*, 2007	
Equivalency Requirement	**Section in Report**
Analysis of external reference material	See Tables A and 10
Results of analyses on reference material from a source different from that used to prepare the calibration standards, if applicable.	See Tables A and 10
Sources of external reference material, if applicable.	See Table 5
Surrogates used, if applicable.	Not Required
Concentrations of surrogates, if applicable.	Not Required
Recoveries of surrogates appropriate to the proposed use, if applicable.	Not Required
Sample preparation.	As per Spectroquant® test kit product flyer or Standard Methods 4500-NH_3 F or report text.
Clean-up procedures.	As per Spectroquant® test kit product flyer or Standard Methods 4500-NH_3 F or report text.
Method blank result.	Table 11
Matrix (reagent water, drinking water, effluent)	Wastewater Effluent
Matrix spikes.	Matrix Spike section the report.
Spiking system, appropriate to the method and application.	Matrix Spike section the report.
Spike concentrations (with units corresponding to the final sample concentration) and recoveries.	Matrix Spike section the report.
Source of spiking material.	Local Muscatine Water Pollution Control Plant
Number of replicate spikes	MS and MSD analyzed and Spectroquant® LFBs. Each sample analyzed spectroscopically and an average and standard deviation determined for the %RSD.
Initial demonstration of capability.	See Table 10
Precision (analyte by analyte) Duplicates.	See Table 9 %RSD

EPA Method Equivalency Check-off Table from Richard Reding Memo, *Flexibility to Modify CWA Methods*, 2007	
Equivalency Requirement	**Section in Report**
Bias (analyte by analyte).	See Table 10
Detection limit (with units; analyte by analyte).	See Table 14
Confirmation of detection limit, if applicable.	See Table 14
Quantitation limit (with units; analyte by analyte) Minimum level (ML), practical quantitation level (PQL) or limit of quantitation (LOQ).	Quantitation limit, Minimum Level, PQL and LOQ set by Spectroquant® product flyer. Tables 9, 10, 11, 12, 13, and 14 confirm that the Spectroquant® test limits in the product flyer are met.
Qualitative confirmation.	Not Required