Method Equivalency Spectroquant® Nitrate Test Kit: Reactions and Photometry

Spectroquant® Test Kits of Merck KGaA, Darmstadt, Germany: 1.00614, 1.01842, 1.09713, 1.14542, 1.14556, 1.14563, 1.14764, 1.14773, 1.14942

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Definitions

1. Nitrate 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 free of nitrate that is used as a zero standard and to calibrate the spectrophotometer
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. 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.
7. 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 Nitrate 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.
8. 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.
9. 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.
10. Water Sample: For the purpose of this method, a sample taken from one of the following sources: drinking water, surface water, 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 Spectroquant® Test Kits 1.00614, 1.01842, 1.09713, 1.14542, 1.14556, 1.14563, 1.147641.14773 and 1.14942 are covered under the method flexibility allowed in the EPA rules 40 CFR part 136.6 [1] The rule lays 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.*
    b. *Changes in reactants provided that the change does not produce interference.*

The method equivalency report for Spectroquant® Test Kits 1.00614, 1.01842, 1.09713, 1.14542, 1.14556, 1.14563, 1.14764, 1.14773 and 1.14942 will directly compare these kits with the allowed method modifications listed in Richard Reading's memo. The MDL results along with the IDC, LRB and LFB and % RSD all show good agreement with the Quality Control parameters that is expected in 40 CFR part 136.7 [1].

This equivalency checklist will directly compare the Spectroquant® nitrate test kits with the simple method modifications listed in Richard Reading's memo and allow a laboratory to establish method equivalency for their analyses and reporting to both users of the results and regulators.

Nitrate Reaction

Chemical Reactions and Spectroscopy

Nitrate Chemistry

The Spectroquant® test kits and all of the Spectroquant® Quality Control (QC) samples identified the active ingredient used in their analyses as Nitrate-N (NO_3^- - N). The Nitrate (NO_3^-) can be calculates from this value by multiplying by a factor of 4.4268

$$NO_3^- - N \times 4.4268 = NO_3^-$$

Figure 1: Nitrate-Nitrogen to Nitrate Conversion Formula

The EPA approved methods that utilize spectroscopic determination identify the active ingredient used in their analyses as Nitrate (NO_3^-) or Nitrate-N (NO_3^- - N). The conversion formula or its reciprocal in Figure 1 is utilized in the EPA results.

Proposed Brucine Nitrate Oxime Azo Dye Reaction

The classical brucine dye reaction in the EPA Method 352.1[3] reaction has been utilized for the detection of nitrate in aqueous samples with in-depth studies published. [4-12] Though no reaction mechanism has been published, the proposed overall reaction proposed utilizing an oxime intermediate produced Weiland-Gumlich aldehyde reaction [13] and sulfanilic acid[14]. The proposed mechanism for the azo dye is formation is illustrated below. (Figure 2)

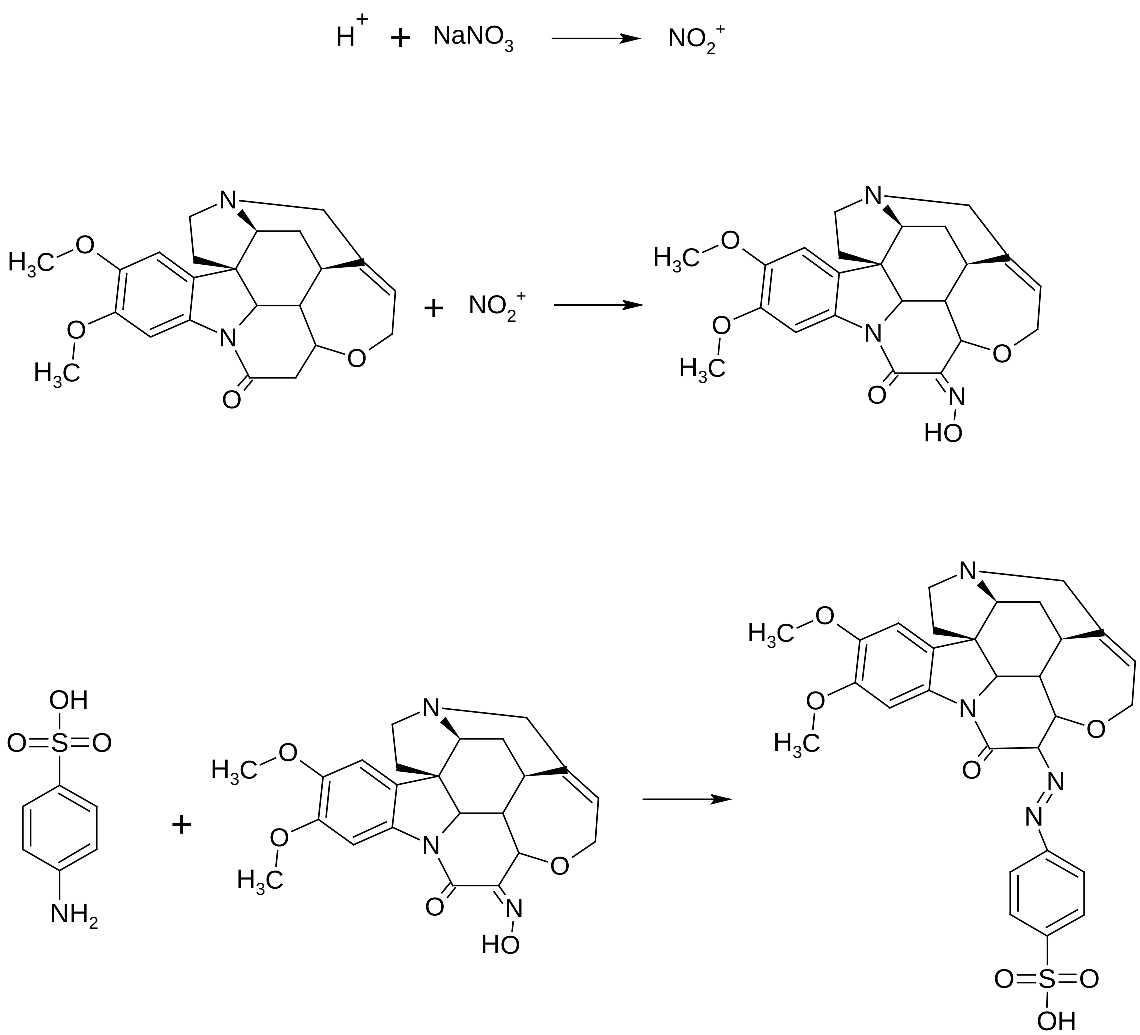

Figure 2: Proposed Brucine Nitrate Oximine Azo Dye Reaction

Classical Cadmium (Cd) Nitrate Reduction Azo Dye Reaction

The classical nitrate reduction to nitrite and the subsequent nitrite-azo dye reaction (Griess Reaction) with sulfanilamide is described in detail in nitrite/nitrate analytical studies [15-20] and in the EPA approved methods in Standard Methods for the Examination of Water and Wastewater 4500 NO_2^-(B) and 4500 NO_3^-(E).[21] The reaction mechanism is detailed in Figure 4 below.

To determine the total amount of nitrate present in solution, the nitrate must be reduced to nitrite. This is achieved when the nitrate in solution flows slowly past a stationary Cadmium-Copper complex. This heterogeneous solid-liquid phase reaction produces nitrite at the discharge of the column.

The nitrite is then determined by the same classical azo dye (Griess Reaction) to determine the concentration of the nitrate. This classical method can only determine the amount of nitrite or nitrite + nitrate quantitatively. If nitrite is present in the sample, then a pre and post Cd-Cu reduction sample is run and the nitrate concentration is determined by the difference of the two results (Figure 3).

Nitrate (mg/L) = (A - B)
A = Concentration of Sample that Contains Suspected Nitrite and Nitrate Run Through Cd-Cu Column
B = Concentration of Sample that Contains Suspected Nitrite and Nitrate Not Run Through Cd-Cu Column
Nitrite (mg/L) = B

Figure 3: Cd-Cu Nitrate and Nitrite Concentration Calculation

$$Cd\text{-}Cu + NO_3^- \longrightarrow NO_2^-$$

$$H^+ + NO_2^- \longrightarrow HNO_2$$

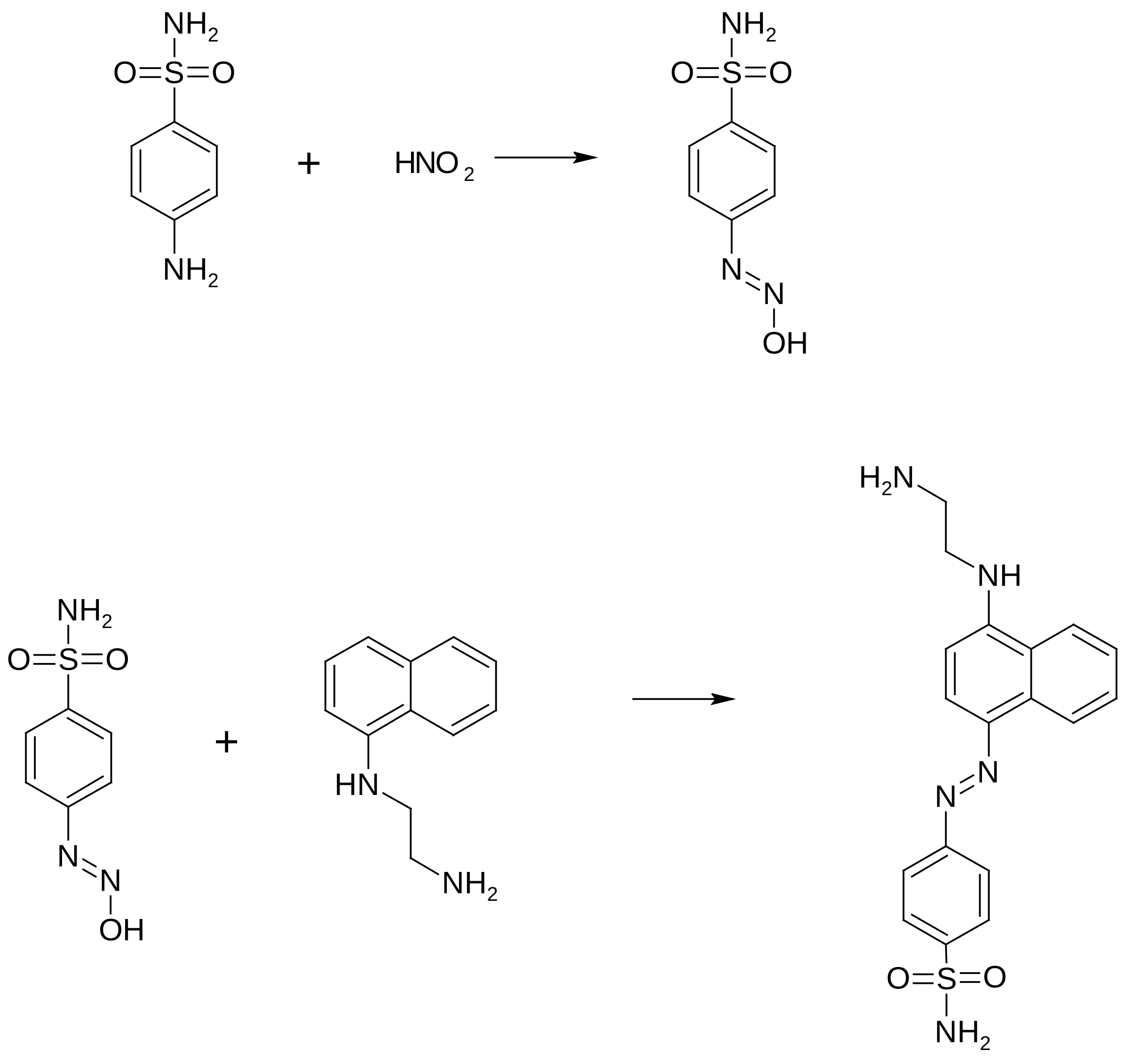

Figure 4: Classical Cadmium (Cd) Nitrate Reduction Azo Dye Reaction

Hach TNT 835-836 Reaction

The Hach test kit utilizes nitrate being converted to the nitronium ion in a concentrated sulfuric acidic solution and then having an electrophilic nitro substitution reaction with a 3,5 Xylenol. This classical electrophilic reaction has been well documented and was utilized by Hach in the EPA Alternate Test Procedure program [22] to recommend or approve this test method for the determination of nitrate in the Clean Water Act (Recommend) [23] or Safe Drinking Water Act (Expedited ATP Approved) [24].

$$H^{+} + NaNO_3 \longrightarrow NO_2^{+}$$

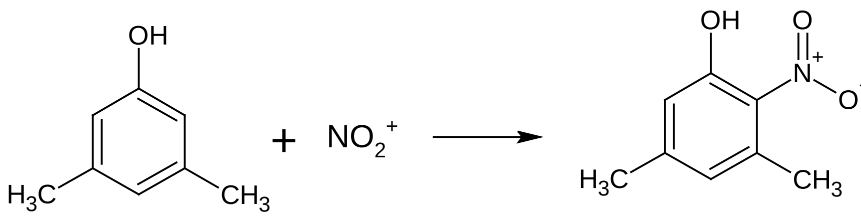

Figure 5: Hach Nitronium Ion-3,5 Xylenol Reaction

Spectroquant® Nitronium Reactions

The Spectroquant® Nitronium reaction with phenolic compounds (2,6 Xylenol, Resorcinol or a Benzoic Acid Derivative) has been identified as a sensitive reaction for nitrate [15, 25-29] and not requiring the multiple steps for colorimetric azo dye formation as required in the EPA Brucine Method 352.1[3]. The reaction mechanism utilizes the nitronium ion produced with concentrated sulfuric acid performing an electrophilic substitution at either the 4 (para) position on the aromatic ring of 2,6 Xylenol or at the 2 (meta), 4 (para) provision on the aromatic ring of Resorcinol or a proprietary reaction with a benzoic acid derivative. .

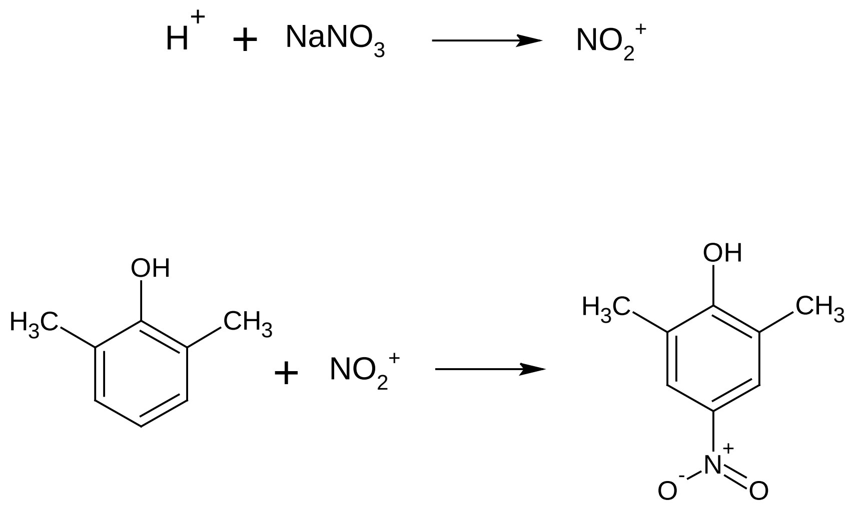

Figure 6: Spectroquant® Nitronium Substitution Reaction, Nitronium Ion and 2,6 Xylenol

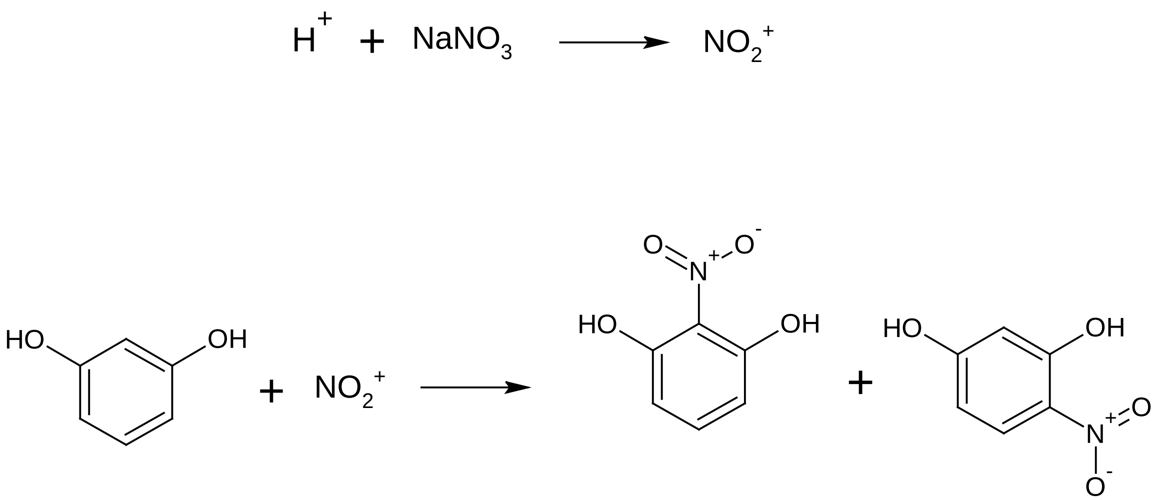

Figure 7: Spectroquant® Nitronium Substitution Reaction, Nitronium Ion and Resorcinol

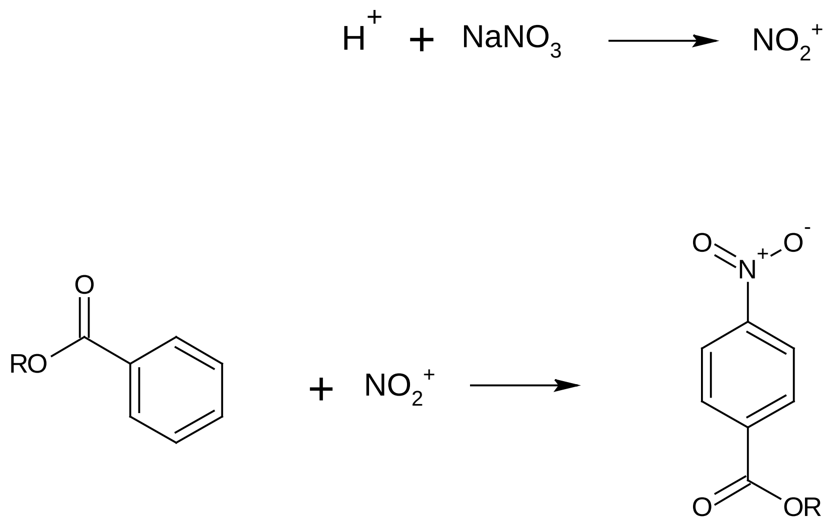

Figure 8: Spectroquant® Nitronium Substitution Reaction, Nitronium Ion and Substituted Benzoic Acid

Spectroquant® Cadmium Reduction Azo Dye Reaction

The Spectroquant® Cadmium Reduction-Azo Dye reaction utilized the ability of Cadmium to reduce nitrate to nitrite and then have the nitrite react to form an azo dye. The exact reactants utilized to produce the final azo dye product are proprietary, but a general mechanism with the formation of a sulfanilic acid intermediate that reacts with a substituted benzoic acid to produce the azo dye product is outlined in Figure 7, below. [30] The absorbance shoulder at 500 nm is utilized with a 50 mm cell to achieve the concentration range listed in the test kit insert.

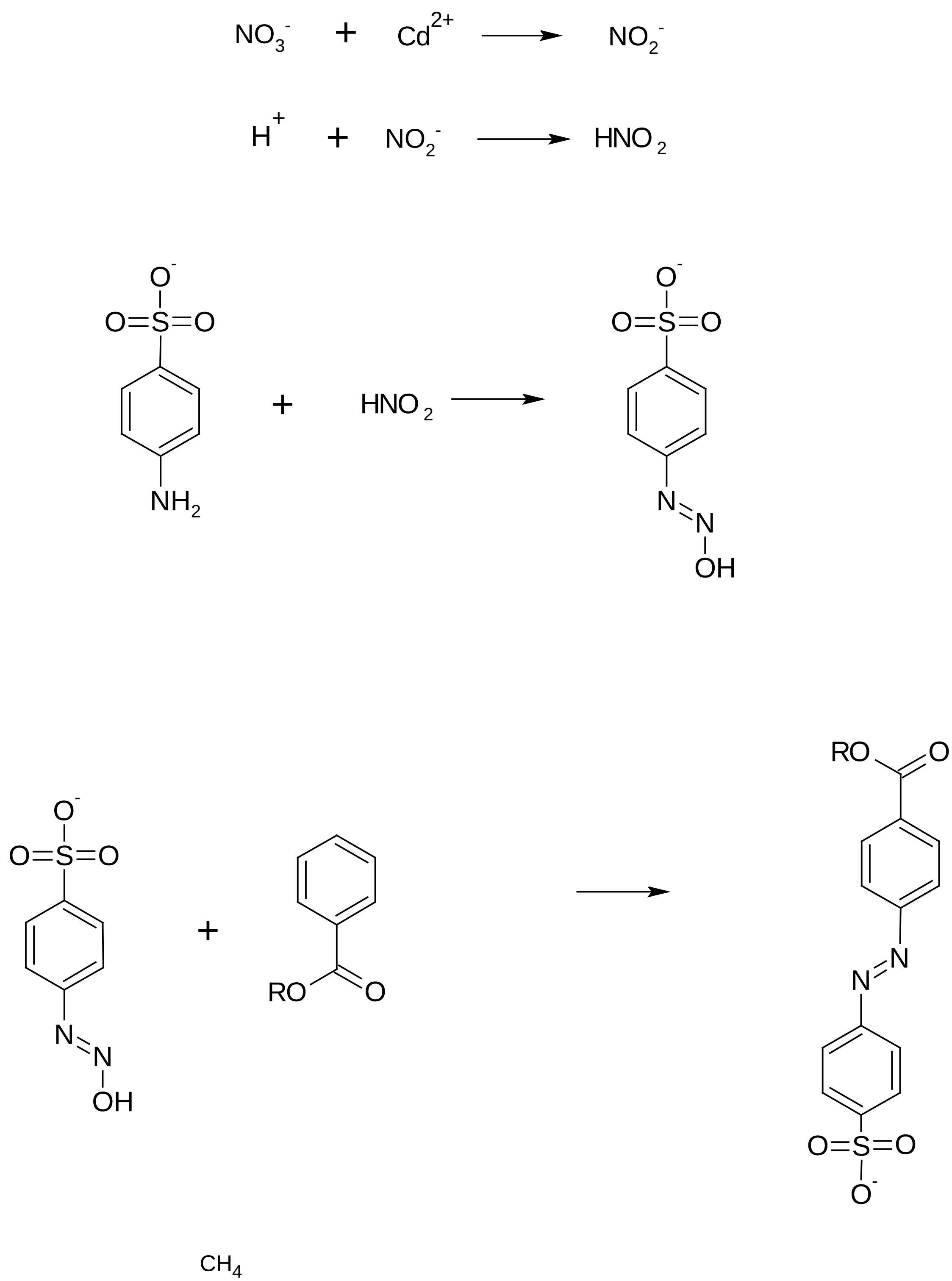

Figure 9: General Spectroquant® Cd Reduction Benzoic Acid Derivative Azo Dye Reaction

The reactants for each Spectroquant® test kit are detailed in Table1 below'

Table 1: Spectroquant® Test Kit Spectrophotometric Reactants		
Spectroquant® Test Kit	Reactant 1	Reactant 2
1.14764	Nitronium Ion	2,6 Xylenol
1.14563	Nitronium Ion	2,6 Xylenol
1.09713	Nitronium Ion	2,6 Xylenol
1.00614	Nitronium Ion	2,6 Xylenol
1.14542	Nitronium Ion	Benzoic Acid Derivative
1.14942	Nitronium Ion	Resorcinol
1.14773	Nitronium Ion	Benzoic Acid Derivative
1.14556	Nitronium Ion	Resorcinol
1.01842	Diazotized Sulfanilic Acid	Benzoic Acid Derivative

Nitrate Reaction-Product Spectra

The Nitronium Ion, Cadmium, Cadmium-Copper absorbance maxima ranges are listed in Table 2 below. The visible spectra for each analytical test are illustrated below (Figures 10-13).

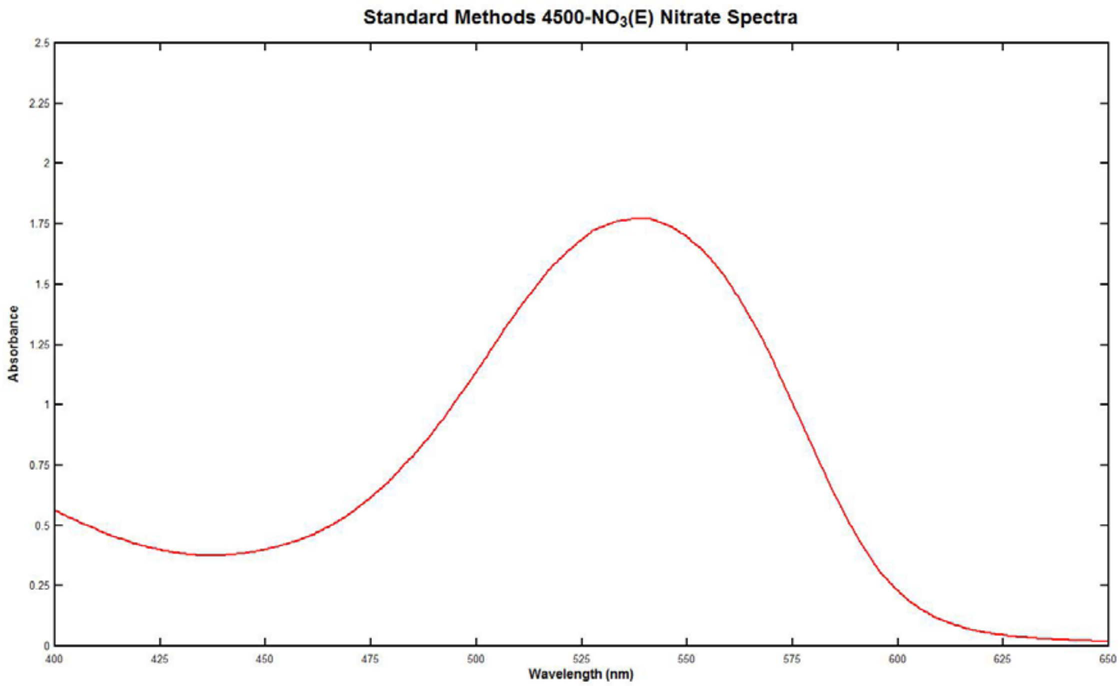

Figure 10: Classical Standard Methods Cd-Cu Reduction 4500 NO_3 (E) Absorbance Spectra

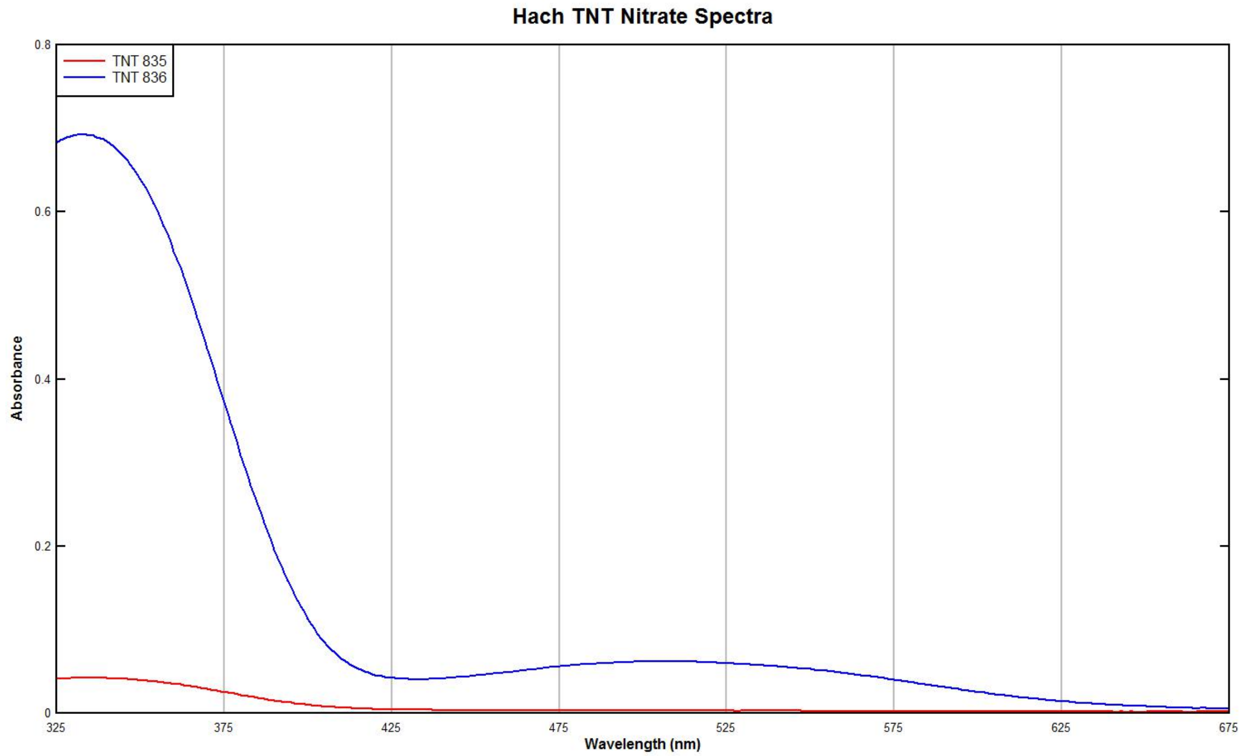

Figure 11: Hach TNT Nitronium Ion 3,5 Xylenol Absorbance Spectra

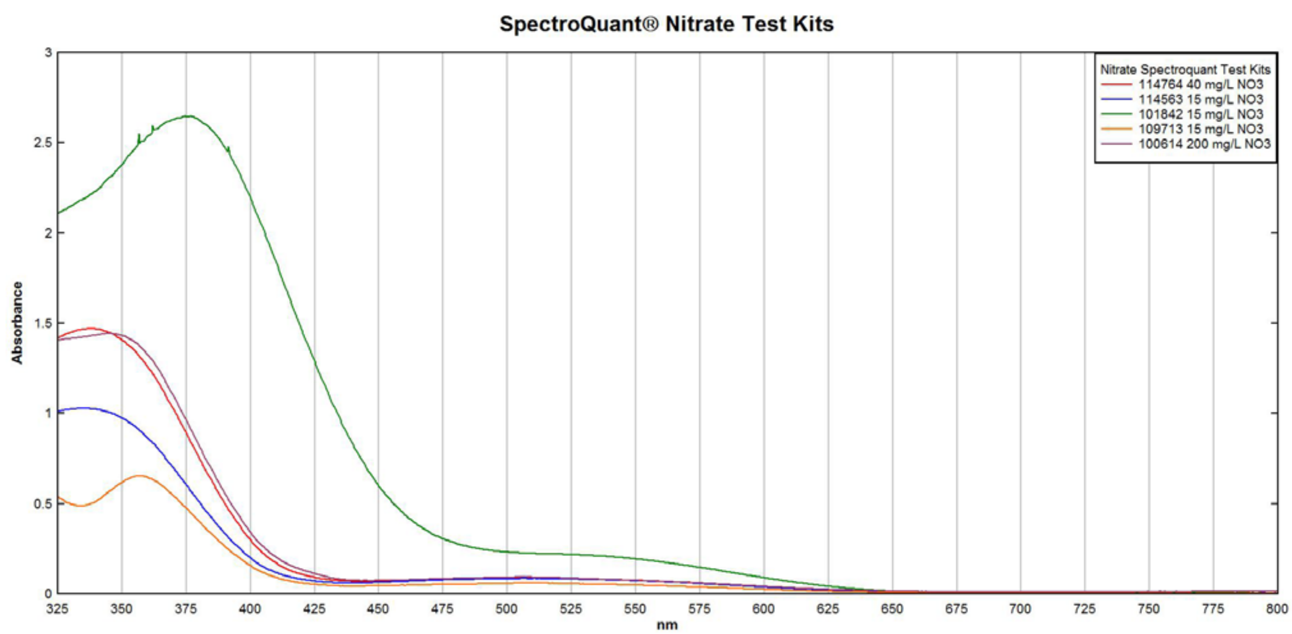

Figure 12: Spectroquant® Test Kits (114764, 114563, 101842, 109713, 100614) Absorbance Spectra

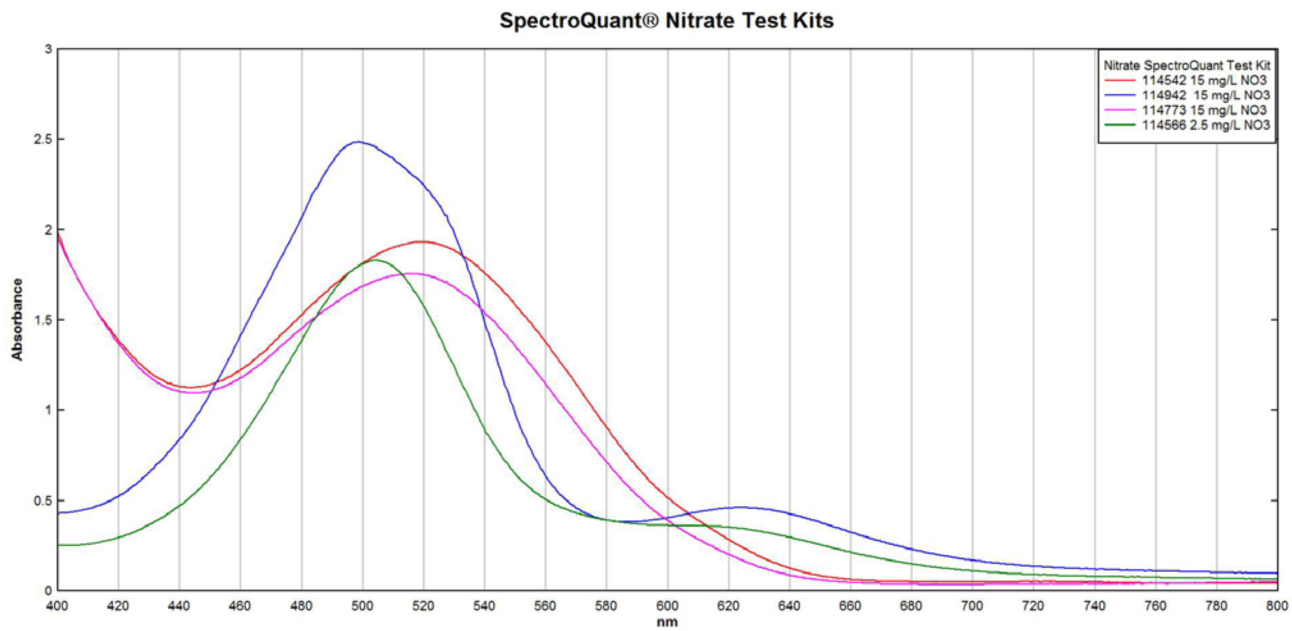

Figure 13: Spectroquant® Test Kit (114542, 114942, 114773, 114566) Absorbance Spectra

Table 2: Spectroquant® Test Kit and Standard Methods Absorbance Maxima Range

nm	1.14764	1.14563	1.09713	1.00614	nm	1.14542	1.14942	1.14773	1.14556	nm	1.01842	nm	TNT 835	TNT 836	nm	Standard Methods
332	1.4592	1.0257	0.49	1.4193	491	1.7019	2.4017	1.5957	1.6773	495	0.2374	325	0.0409	0.6827	533.5	1.7610
332.5	1.4611	1.0265	0.4886	1.4201	491.5	1.7078	2.4111	1.6007	1.6864	495.5	0.237	326	0.0413	0.6857	534	1.7627
333	1.4627	1.0267	0.4881	1.4205	492	1.714	2.4195	1.6057	1.6945	496	0.2361	327	0.0414	0.6875	534.5	1.7639
333.5	1.4642	1.027	0.4877	1.4214	492.5	1.7221	2.4381	1.6135	1.7049	496.5	0.2352	328	0.0416	0.6893	535	1.7651
334	1.4655	1.0272	0.4878	1.4222	493	1.7286	2.443	1.619	1.7161	497	0.2345	329	0.0418	0.6907	535.5	1.7666
334.5	1.4663	1.0273	0.4884	1.4238	493.5	1.7358	2.4486	1.625	1.7255	497.5	0.2338	330	0.0418	0.6915	536	1.7684
335	1.467	1.0271	0.489	1.4242	494	1.7431	2.4547	1.631	1.7351	498	0.2335	331	0.0419	0.6922	536.5	1.7696
335.5	1.4679	1.0271	0.4902	1.4248	494.5	1.7503	2.4619	1.637	1.7438	498.5	0.2327	332	0.0421	0.6925	537	1.7708
336	1.4682	1.0269	0.4914	1.4259	495	1.7579	2.4663	1.6425	1.7549	499	0.2322	333	0.042	0.6924	537.5	1.7705
336.5	1.4688	1.0265	0.4935	1.4274	495.5	1.7642	2.4728	1.6474	1.7615	499.5	0.2316	334	0.0422	0.6924	538	1.7702
337	1.4694	1.0263	0.4956	1.4287	496	1.7704	2.4759	1.6517	1.7688	500	0.2311	335	0.0422	0.6916	538.5	1.7699
337.5	1.4696	1.0259	0.4986	1.4297	496.5	1.7753	2.4817	1.6566	1.775	500.5	0.2307	336	0.0421	0.691	539	1.7698
338	1.4693	1.0253	0.5012	1.4311	497	1.7816	2.4832	1.6608	1.7817	501	0.2298	337	0.0421	0.6897	539.5	1.7699
338.5	1.4693	1.0249	0.504	1.4327	497.5	1.7875	2.4829	1.6656	1.7875	501.5	0.2297	338	0.042	0.6883	540	1.7704
339	1.4691	1.0242	0.5077	1.4332	498	1.7931	2.4844	1.6702	1.7942	502	0.2289	339	0.042	0.6866	540.5	1.7704
339.5	1.4686	1.0237	0.5117	1.4343	498.5	1.7995	2.4844	1.6754	1.7992	502.5	0.2283	340	0.0419	0.6844	541	1.7691
340	1.4678	1.0226	0.5155	1.4356	499	1.8054	2.4849	1.6795	1.8035	503	0.2282	341	0.0417	0.6818	541.5	1.7690
340.5	1.4667	1.0213	0.5199	1.4364	499.5	1.8104	2.4838	1.6834	1.8076	503.5	0.2279	342	0.0415	0.6784	542	1.7673
341	1.4655	1.0202	0.5248	1.4377	500	1.816	2.4813	1.6871	1.8118	504	0.2272	343	0.0414	0.6745	542.5	1.7652
341.5	1.4645	1.0194	0.5297	1.4392	500.5	1.8216	2.4777	1.6915	1.8163	504.5	0.227				543	1.7621
342	1.4627	1.0175	0.5355	1.4398	501	1.8267	2.4751	1.6951	1.8184	505	0.2265				543.5	1.7583
342.5	1.461	1.0157	0.5413	1.4407	501.5	1.8311	2.4754	1.6987	1.8214							
343	1.4586	1.0139	0.5471	1.4417	502	1.8367	2.4722	1.7024	1.8245							

Table 2: Spectroquant® Test Kit and Standard Methods Absorbance Maxima Range

nm	1.14764	1.14563	1.09713	1.00614	nm	1.14542	1.14942	1.14773	1.14556	nm	1.01842	nm	TNT 835	TNT 836	nm	Standard Methods
343.5	1.4564	1.0118	0.5524	1.442	502.5	1.8418	2.4702	1.7064	1.8262							
344	1.4545	1.0102	0.558	1.443	503	1.8473	2.4644	1.7095	1.8283							
344.5	1.452	1.0082	0.563	1.4432	503.5	1.8523	2.4626	1.7131	1.8292							
345	1.4491	1.0058	0.5682	1.4431	504	1.8577	2.457	1.7172	1.8302							
345.5	1.4461	1.0036	0.5736	1.4428	504.5	1.8615	2.4546	1.7202	1.8298							
346	1.4431	1.0012	0.5788	1.4423	505	1.8657	2.4468	1.7229	1.8291							
346.5	1.4392	0.998	0.5841	1.4416	505.5	1.8702	2.4415	1.7256	1.8281							
347	1.4351	0.9958	0.5888	1.4403	506	1.875	2.4361	1.7292	1.8259							
347.5	1.4307	0.9923	0.5946	1.4402	506.5	1.8795	2.4284	1.7315	1.8242							
348	1.4263	0.9889	0.6004	1.439	507	1.8824	2.4248	1.7337	1.8216							
354	1.3601	0.9398	0.6476	1.4072	507.5	1.8857	2.4195	1.7363	1.8183							
354.5	1.3525	0.9342	0.6494	1.402	508	1.889	2.4139	1.7388	1.8151							
355	1.3448	0.9285	0.6512	1.3961	508.5	1.8923	2.4091	1.7405	1.8106							
355.5	1.3397	0.9226	0.6524	1.3906	509	1.8971	2.4031	1.7425	1.8068							
356	1.3282	0.9161	0.6529	1.3857	509.5	1.8995	2.3983	1.7442	1.801							
356.5	1.3203	0.9103	0.6546	1.378	510	1.903	2.3925	1.7447	1.7957							
357	1.3126	0.9046	0.6524	1.3717	510.5	1.9055	2.3854	1.7476	1.7889							
357.5	1.3054	0.8991	0.6527	1.366	511	1.9085	2.3783	1.7493	1.781							
358	1.2973	0.8932	0.6518	1.3589	511.5	1.9104	2.3714	1.7503	1.7741							
358.5	1.288	0.8867	0.6509	1.3513	512	1.914	2.3653	1.7514	1.7672							
359	1.2776	0.879	0.6489	1.3421	512.5	1.9163	2.3582	1.7522	1.759							
					513	1.9179	2.3529	1.753	1.7492							
					513.5	1.9198	2.3428	1.7543	1.7395							

Table 2: Spectroquant® Test Kit and Standard Methods Absorbance Maxima Range

nm	1.14764	1.14563	1.09713	1.00614	nm	1.14542	1.14942	1.14773	1.14556	nm	1.01842	nm	TNT 835	TNT 836	nm	Standard Methods
					514	1.9208	2.3377	1.7547	1.7301							
					514.5	1.9234	2.3305	1.7557	1.718							
					515	1.9256	2.3196	1.7548	1.7059							
					515.5	1.9263	2.3148	1.756	1.6942							
					516	1.9288	2.3061	1.7556	1.6814							
					516.5	1.9295	2.2988	1.7557	1.6667							
					517	1.9302	2.2934	1.7555	1.6569							
					517.5	1.9308	2.2873	1.7549	1.6444							
					518	1.9321	2.2815	1.7542	1.6318							
					518.5	1.9319	2.2731	1.7534	1.6196							
					519	1.9323	2.2666	1.7527	1.6068							
					519.5	1.9325	2.2555	1.751	1.5892							
					520	1.9322	2.2456	1.7501	1.5767							
					520.5	1.9311	2.236	1.7488	1.562							
					521	1.9311	2.2249	1.7469	1.5451							
					521.5	1.9295	2.2151	1.7447	1.5284							
					522	1.9289	2.2052	1.7427	1.5139							
					522.5	1.927	2.1947	1.7401	1.4969							
					523	1.9267	2.1825	1.7377	1.4806							
					523.5	1.9247	2.1733	1.7347	1.4655							
					524	1.923	2.1603	1.7319	1.4477							
					524.5	1.921	2.1507	1.7285	1.4313							
					525	1.9193	2.139	1.7249	1.4147							

Table 2: Spectroquant® Test Kit and Standard Methods Absorbance Maxima Range

nm	1.14764	1.14563	1.09713	1.00614	nm	1.14542	1.14942	1.14773	1.14556	nm	1.01842	nm	TNT 835	TNT 836	nm	Standard Methods
					525.5	1.9162	2.1258	1.7213	1.3971							
					526	1.9139	2.1118	1.7178	1.3799							
					526.5	1.9107	2.0969	1.7135	1.3616							
					527	1.9068	2.0785	1.7077	1.3404							
					527.5	1.9055	2.0771	1.7124	1.3237							
					528	1.9005	2.0462	1.6971	1.3059							

Analytical Standards

For this study, all analytical standards were prepared as a NO_3^--N. The nitrate standard was prepared from $NaNO_3$ (Sigma S5506, Assay 99.5 %) which was dried overnight at 104 ºC and stored in a desiccator until cool. This sodium nitrate was then used within 8 hours of cooling to prepare the standards listed in Tables 3-4 below.

Table 3: Nitrate Standard Stock 1 as NO_3^- - N	
1000 mg/L Standard Stock Solution 1 Preparation	
MW Nitrate Standard (g)	84.9900
MW N (g)	14.0067
Mole Fraction N	0.1648
Grams $NaNO_3$ to Provide 1000 mg/$NaNO_3$ - N	6.0678
Purity $NaNO_3$ Sigma Aldrich Lot# MKBC5139	0.995
Corrected Grams $NaNO_3$ to Provide 1000 mg/$NaNO_3$ - N	6.0983

1. Standard Stock Solution 1 (1000 mg/L NO_3^- - N):
   1.1. 6.0983 g (± 0.0001 g) of the dried sodium nitrate was dissolved in 800 mL of nitrate free DI water in a Class A 1000 mL volumetric flask.
   1.2. Nitrate free water was added to the mark (1000 mL).
   1.3. Stock Solution 1 was mixed completely by flask inversion (Minimum 20 times).
2. Standard Stock Solution 2 (500 mg/L NO_3^- - N):
   2.1. 500 mL of Standard Stock Solution 1 was transferred with a Class A 50 mL pipet to a Class A 1000 mL volumetric flask.
   2.2. Nitrate free DI water was added to the mark (1000 mL).
   2.3. Stock Solution 2 was mixed completely by flask inversion. (Minimum 20 times)
3. Standard Stock Solution 3 (100 mg/L NO_3^- - N):
   3.1. 100 mL of Standard Stock Solution 1 was transferred with a Class A 50 mL pipet to a Class A 1000 mL volumetric flask.
   3.2. Nitrate free DI water was added to the mark (1000 mL).
   3.3. Stock Solution 3 was mixed completely by flask inversion (Minimum 20 times).
4. Standard Stock Solution 4 (10 mg/L NO_3^- - N):
   4.1. 100 mL of Standard Stock Solution 3 was transferred with a Class A 50 mL pipet to a Class A 1000 mL volumetric flask.

4.2. Nitrate free DI water was added to the mark (1000 mL).

4.3. Stock Solution 4 was mixed completely by flask inversion (Minimum 20 times).

5. Standard Stock Solution 5 (1.0 mg/L NO_3^- - N):

5.1. 100 mL of Standard Stock Solution 4 was transferred with a Class A 50 mL pipet to a Class A 1000 mL volumetric flask.

5.2. Nitrate free DI water was added to the mark (1000 mL).

5.3. Stock Solution 5 was mixed completely by flask inversion (Minimum 20 times).

6. Standard Stock Solution 6 (0.1 mg/L NO_3^- - N):

6.1. 100 mL of Standard Stock Solution 5 was transferred with a Class A 50 mL pipet to a Class A 1000 mL volumetric flask.

6.2. Nitrate free DI water was added to the mark (1000 mL).

6.3. Stock Solution 6 was mixed completely by flask inversion (Minimum 20 times).

7. All other standards were prepared from Stock Standards following the dilution volumes listed in Table 3.

7.1. The Stock Standard volume listed in Table 3 was transferred with a Class A volumetric pipet to a Class A 100 mL volumetric flask.

7.2. Nitrate free DI water was added to the mark (100 mL).

7.3. Standard was mixed completely by flask inversion (Minimum 20 times).

8. Fresh standards were prepared every 14 days.

Table 4: Nitrate Standard Preparation

Stock Standard	**Volume of Stock Standard Diluted to 100 mL**	**Final Standard Concentration**
1		
2	45	225
2	40	200
2	30	150
2	25	125
3	100	100
3	75	75
3	50	50
3	30	30
3	25	25
3	20	20

Table 4: Nitrate Standard Preparation		
Stock Standard	**Volume of Stock Standard Diluted to 100 mL**	**Final Standard Concentration**
3	23	23
3	18	18
3	17	17
3	15	15
3	12	12
4	100	10
4	75	7.5
4	50	5
4	30	3
4	20	2
5	50	0.5
5	30	0.3
5	20	0.2
5	10	0.1

Other Sample Preparation

The samples used in this method equivalency study were either standards prepared in nitrate free DI water, Spectroquant® Check Samples(Table 8) or Final Effluent water. The Final Effluent was filtered through a 0.45 µm Whatman PES syringe filter to remove turbidity.

The Standard Methods for the Examination of Water and Wastewater [21] methods approved by EPA [1] allows the analyses of these nitrate samples with filtration if needed. Filtration is identified in the Spectroquant® product flyers and is acceptable under the EPA approved method 4500-NO_3^- A-2000 (2011).

Table 5: Test Kit and Standard Method Maxima Wavelengths	
Test Kit or Method	**Quantitation Wavelength (nm)**
1.14764	337
1.14563	334
1.09713	357
1.00614	345

Table 5: Test Kit and Standard Method Maxima Wavelengths	
1.14542	520
1.14942	499
1.14773	516
1.14556	504
1.01842	500
TNT 835	335
TNT 836	335
Standard Methods 4500-NO_3 (E)	537

Calibration and Linear/Quadratic Correlation Coefficient

Utilizing both the absorbance maximum determined experimentally for the Spectroquant® test kit, Hach TNT test kits and Standard Methods 4500-NO_3 (E), the Thermo Scientific Genesys 10S was calibrated with a minimum of six (6) standards and a linear or quadratic correlation coefficient was determined. The standards bracketed the Spectroquant® test kit concentrations listed in the product flyer.

The correlation coefficient was >0.997 in all cases indicating that the Spectroquant® Test Kits can produce an acceptable linear or quadratic calibration. The calibration standards using mg/L (NO_3-N) for each test kit along with the linear or quadratic Correlation Coefficients are summarized in Tables 6-7 below.

Table 6: Spectroquant® Test Kit Calibration Standards and Correlation Coefficient										
Test Kit	**Wavelength (nm)**	**Standard 1***	**Standard 2***	**Standard 3***	**Standard 4***	**Standard 5***	**Standard 6***	**Standard 7***	**Standard 8***	**Correlation Coefficient**
										Linear Intercept Through Zero 0.99997
1.14764	337	0.00	1.00	5.00	10.00	15.00	25.00	30.00	50.00	
										Linear Intercept Through Zero 0.999954
1.14563	334	0.00	0.50	1.00	3.00	5.00	10.00	15.00	25.00	
										Linear Intercept Through Zero 0.99977
1.09713	357	0.00	1.00	3.00	5.00	10.00	15.00	20.00	25.00	
										Linear Intercept Through Zero 0.99999
1.00614	345	0.00	23.00	30.00	50.00	100.00	150.00	200.00	225.00	
										Linear Intercept Through Zero 0.99794
1.14542	520	0.00	0.50	1.00	3.00	5.00	10.00	15.00	18.00	
										Quadratic, Intercept Calculated 0.99952
1.14942	499	0.00	0.20	0.50	1.00	5.0	10.00	15.00	17.00	
										Linear Intercept Through Zero 0.99933
1.14773	516	0.00	0.20	0.50	2.00	5.00	10.00	15.00	20.00	
										Quadratic Intercept Calculated 0.99914
1.14556	504	0.00	0.10	0.30	0.50	1.00	2.00	3.00		

Table 6: Spectroquant® Test Kit Calibration Standards and Correlation Coefficient										
Test Kit	**Wavelength (nm)**	**Standard 1***	**Standard 2***	**Standard 3***	**Standard 4***	**Standard 5***	**Standard 6***	**Standard 7***	**Standard 8***	**Correlation Coefficient**
										Quadratic Intercept Through Zero 0.99868
1.01842	500	0.00	0.30	0.50	1.00	5.00	10.00	25.00	30.00	

* Standard Concentration NO_3-N

Table 7: Standard Methods 4500-NO_3 (E) and Hach TNT Calibration Standards and Correlation Coefficient									
Test Kit	**Wavelength (nm)**	**Standard 1***	**Standard 2***	**Standard 3***	**Standard 4***	**Standard 5***	**Standard 6***	**Standard 7***	**Correlation Coefficient**
									Quadratic, Intercept Calculated 0.99930
Standard Methods 4500-NO_3 (E)	**537**	0.000	0.200	0.300	0.500	1.000			
									Quadratic, Intercept Calculated 0.99823
Hach TNT 835	**335**	0.000	0.300	0.500	1.000	5.000	10.000	12.000	
									Quadratic, Intercept Calculated 0.99984
Hach TNT 836	**335**	0.000	5.000	10.000	15.000	20.000	25.000	30.000	

* Standard Concentration NO_3^-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 nitrate. The control check concentration (accuracy) and precision are summarized in Tables 9-10 below. The percent recoveries for each Spectroquant® test kit, Hach TNT test kit and Standard Methods 4500-NO_3 (E) [21] 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 14: 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).[31-33]

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

Figure 15: Percent Relative Standard Deviation

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

Spectroquant® Check Sample	Label Concentration (NO_3^--N mg/L)	Check Sample Actual Concentration (NO_3^--N mg/L)	Check Sample Precision (± mg/L)
1.25036.0100	0.50	0.49	0.05
1.25037.0100	2.50	2.48	0.06
1.25038.0100	15.0	14.9	0.4
1.25039.0100	40.0	40.2	1.0
1.25040.0100	200	200	5

The % RSD was calculated for the Spectroquant®, Hach TNT and Standard Methods 4500-NO_3 (E) [21] check samples that were used for the accuracy evaluation. The number of replicate concentration determinations at the maximum wavelength (nm) determined for each

Spectroquant® test kit, Hach TNT test kit and 4500-NO_3 (E) 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.

The accuracy or recovery of the Spectroquant® check samples by either the Spectroquant® test kits, Hach TNT test kit or 4500-NO_3 (E) was no lower than 86.29 % and no higher than 102.62 %. The % RSD of the Spectroquant® check samples by the Spectroquant® test kits, Hach TNT test kit 6 or the classical phenate method was no greater than 6.962 %..

These efficient recoveries of Spectroquant® check samples and the low % RSD shows that the Spectroquant® will produce equivalent accurate and precise results when compared to Standard Methods 4500-NO_3 (E) [21].

Table 9: LFB Recovery Comparison												
Spectroquant® Check Sample	**Label Concentration (NO_3-N mg/L)**	**Standard Methods 4500-NO_3 (E)**	**Hach TNT 836**	**1.14764**	**1.14563**	**1.09713**	**1.00614**	**1.14542**	**1.14942**	**1.14773**	**1.14556**	**1.01842**
1.25036.0100	0.50	99.49									91.84	
1.25037.0100	2.50			98.79	100.81	99.19		97.98	86.29	100.00	100.00	
1.25038.0100	15.0		99.47	99.13	99.87	99.13		97.99	100.81	100.94		102.62
1.25039.0100	40.0						98.91					
1.25040.0100	200						97.99					

Table 10: % RSD Comparison												
Spectroquant® Check Sample	**Label Concentration (NO_3-N mg/L)**	**Standard Methods 4500-NO_3 (E)**	**Hach TNT 836**	**1.14764**	**1.14563**	**1.09713**	**1.00614**	**1.14542**	**1.14942**	**1.14773**	**1.14556**	**1.01842**
1.25036.0100	0.50	0.931									5.917	
1.25037.0100	2.50			1.633	0.596	4.633		6.962	1.966	3.077	2.683	
1.25038.0100	15.0		2.037	0.897	1.848	0.415		0.965	1.028	1.269		0.065
1.25039.0100	40.0						0.315					
1.25040.0100	200						2.988					

Laboratory Reagent Blank (LRB)

A volume of DI reagent water 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 as seven (7) unique samples with five replicates per sample for each Spectroquant® test and the average is reported in Table 11 below. In all cases, nitrate was not seen at a level above 0.106 mg/L NO_3-N

Table 11: Spectroquant® Laboratory Reagent Blank (mg/L NO_3-N)

1.14764	1.14563	1.09713	1.00614	1.14542	1.14942	1.14773	1.14556	1.01842
0.034	0.006	0.003	0.106	-0.030	-0.009	0.000	0.071	-0.010

Initial Demonstration of Capability (IDC)

Control samples of nitrate were prepared and analyzed with each Spectroquant® Test Kit. The IDC was calculated from the average of 4 control samples run 5 times each and the standard deviation based on the formula in Standard Methods 4020 [21].

$$\text{IDC Limits} = \text{Average} \pm (\text{Standard Deviation} \times 5.84)$$

Figure 16: IDC Calculation for 4 Control Samples

The results are summarized in Table 12 below. Each IDC analysis value for each test kit was between upper and lower limits calculated for a passing IDC. All IDC samples passed for each Spectroquant® Test Kit, Hach TNT test kit and Standard Methods 4500-NO_3 (E) [21]..

Table 12: Initial Demonstration of Capability (IDC)

IDC Values (mg/L NO3-N)	Average	IDC Low	IDC High	Average	IDC Low	IDC High	Average	IDC Low	IDC High	Average	IDC Low	IDC High	Average	IDC Low	IDC High
Standard Methods 4500-NO_3 (E)	0.491	0.465	0.518												
Hach TNT 836							15.015	13.299	16.801						
1.14764				2.500	2.260	2.740	14.913	14.131	15.694						
1.14563				2.520	2.430	2.610	14.790	13.193	16.387						

Table 12: Initial Demonstration of Capability (IDC)															
IDC Values (mg/L NO3-N)	**Average**	**IDC Low**	**IDC High**	**Average**	**IDC Low**	**IDC High**	**Average**	**IDC Low**	**IDC High**	**Average**	**IDC Low**	**IDC High**	**Average**	**IDC Low**	**IDC High**
1.09713				2.550	1.860	3.230	14.860	14.500	15.220						
1.00614										39.833	39.099	40.566	196.563	162.265	230.860
1.14542				2.390	1.420	3.350	14.530	13.711	15.349						
1.14942				2.090	1.850	2.330	15.018	14.116	15.919						
1.14773				2.440	2.000	2.880	14.973	13.863	16.082						
1.14556	0.423	0.277	0.569	2.420	2.040	2.800									
1.01842							15.295	15.237	15.353						

Matrix Spikes

A Final Effluent 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 the Final Effluent was obtained from the Muscatine Water Pollution Control Plant in Muscatine, Iowa. This sample was then filtered through a 0.45 µm syringe filter prior to matrix analyses.

The Muscatine Water Pollution Control laboratory has indicated that nitrate concentrations in the in the Final Effluent was in the range 0.5 to 1.5 mg/L. The Spectroquant® test kit 1.14556, 1.14563 and 1.01842 were selected for the test as they are representative of the different wavelength ranges or reactant chemistries..

Each spectroscopic analysis was measured five (5) times and the average is reports and the standard deviation was used in the % RSD calculation.

Figures 17-18 provide the calculations used. The percent recovery, RPD and % RSD for the samples are summarized in Table 13-14 below.

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

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

Figure 17: 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 18: Relative Percent Difference (RPD)

The percent recovery of the matrix spikes was between 89% and 107%. The RPD was between 0.35% and 1.69%. The %RSD was no greater than 1.11%

The results show that the Spectroquant® test kit can determine nitrate in a matrix regulated under the Clean Water Act (CWA)

Table 13: Spectroquant® Matrix Spike Percent Recovery and RPD (2.00 mNO_3-N mg/L)			
	1.14556	**1.14563**	**1.01842**
Final Effluent (NO_3-N mg/L)	0.58	0.82	1.46
Matrix Spike (NO_3-N mg/L)	2.35	2.87	3.53
Matrix Spike Percent Recovery	89%	103%	104%
Matrix Spike Duplicate (NO_3-N mg/L)	2.39	2.86	3.59
Matrix Spike Duplicate Percent Recovery	91%	102%	107%
Relative Percent Difference	1.69%	0.35%	1.69%

Table 14: Spectroquant® Final Effluent, Matrix Spike and Spike Duplicate % RSD			
	1.14556	**1.14563**	**1.01842**
Final Effluent	0.00%	0.00%	0.68%
Matrix Spike	0.00%	0.00%	0.56%
Matrix Spike Duplicate	0.00%	0.00%	1.11%

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 [34]. Each Spectroquant® test kit product flyer and test kit contained a nitrate range for which the test kit reagents were at a concentration to produce complete reaction of nitrate. 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 can provide a chromophore that responds like the Standard Methods 4500-NO_3 (E) method and that the dynamic range listed on each Spectroquant® product flyer is accurate.

Table 15: Spectroquant® Method Reporting Limit - Method Detection Limits			
Spectroquant® Test Kit	**Spectrometer Cell Width (mm)**	***MRL Lowest Concentration Listed for Spectroquant® Test Kit (NO_3-N)***	**MDL (NO_3-N)**
1.14764	**10**	***1.00***	**0.456**
1.14563	**10**	***0.50***	**0.114**
1.09713	**10**	***1.00***	**0.120**
1.00614	**10**	***23.00***	**2.101**
1.14542	**10**	***0.50***	**0.001**
1.14942	**10**	***0.20***	**0.136**
1.14773	**10**	***0.50***	**0.040**
1.14556	**10**	***0.10***	**0.073**
1.01842	**50**	***0.30***	**0.159**

Spectroquant®-EPA Equivalent Methods (Test Kits 1.14764, 1.14563, 1.09713, 1.00614, 1.14542, 1.14942, 1.14773, 1.14556)

An overall review of the nitrate test kits 1.14764, 1.14563, 1.09713, 1.00614, 1.14542, 1.14942, 1.14773 and 1.14556 spectrophotometric analytical reagents and their equivalency to EPA promulgated method are summarized in Table 16. 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. 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.
    b. Changes in reactants provided that the change does not produce interference.

The chemistry and analytical procedure for Spectroquant® Test Kits 1.14563, 1.09713, 1.00614, 1.14542, 1.14942, 1.14764, 1.14773 and 1.14556 have:

1. A nitronium ion produced from nitrate similar to the EPA method 352.1
2. Instead of an azo dye being formed as with brucine, the nitronium ion performs electrophilic substitution with to attach a nitro group to 2,6 Xylenol, Resorcinol or a substituted benzoic acid to produce the final photomer.
3. The MDL results along with the IDC, LRB, LFB, MS/MSD, RPD and % RSD all show good agreement with the Quality Control parameters that is expected in 40 CFR part 136.7 [1].

Spectroquant®-EPA Equivalent Method (Test Kit 1.01842)

An overall review of the nitrate test kit 1.01842 spectrophotometric analytical reagents and their equivalency to EPA promulgated method are summarized in Table 12. 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. 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.
    b. Changes in reactants provided that the change does not produce interference.

The chemistry and analytical procedure for Spectroquant® Test Kits 1.01842 has:

1. Nitrite produced from nitrate reaction with Cadmium similar to the 4500-NO_3^- (E)
2. An azo dye being formed as with a substituted benzoic acid to produce the final photomer.
3. The MDL results along with the IDC, LRB, LFB, MS/MSD, RPD and % RSD all show good agreement with the Quality Control parameters that is expected in 40 CFR part 136.7 [1].

Table 16: Spectroquant® Test Kit Method Equivalency Summary		
Spectroquant® Test Kit	Spectroquant® Change	136.6 Requirement
1.14764, 1.14563, 1.09713, 1.00614, 1.14542, 1.14942, 1.14773, 1.14556	Nitronium Ion Formation and Electrophilic Substitution to Add a Nitro Group to 2,6 Xylenol, Resorcinol or a Substituted Benzoic Acid	Changes Allowed Under Method Equivalency. • Changes in complexing reagent provided that the change does not produce interferences. • Changes in reactants provided that the change does not produce interference. • Accuracy, Precision, MRL and MDL from the experimental data were acceptable.
1.14764, 1.14563, 1.09713, 1.00614, 1.14542, 1.14942, 1.14773, 1.14556	Absorbance Maxima Changed	Changes Allowed Under Method Equivalency. • Changes in equipment operating parameters such as minor changes in the monitoring wavelength of a colorimeter • Accuracy, Precision, MRL and MDL from the experimental data were acceptable.

<table>
<tr><th colspan="3">Table 16: Spectroquant® Test Kit Method Equivalency Summary</th></tr>
<tr><td>Spectroquant® Test Kit</td><td>Spectroquant® Change</td><td>136.6 Requirement</td></tr>
<tr><td rowspan="2">1.00609</td><td>Azo Dye Reaction with Substituted Benzoic Acid</td><td>Changes Allowed Under Method Equivalency.
Minor changes in reagents used where the underlying reaction and principles remain virtually the same:
Changes in complexing reagent provided that the change does not produce interferences.
Changes in reactants provided that the change does not produce interference.
Accuracy, Precision, MRL and MDL from the experimental data were acceptable.</td></tr>
<tr><td>Absorbance Maxima Changed</td><td>Changes Allowed Under Method Equivalency.
Changes in equipment operating parameters such as minor changes in the monitoring wavelength of a colorimeter
Accuracy, Precision, MRL and MDL from the experimental data were acceptable.</td></tr>
</table>

Summary and Conclusion

Summary

This study has provided the literature review information, EPA 40 CFR part 136.6 requirements and experimental data to support that Spectroquant® Nitrate Tests Kits 1.14764, 1.14563, 1.09713, 1.00614, 1.14542, 1.14942, 1.14773, 1.14556 and 1.00609 are equivalent to the EPA promulgated methods.

Conclusion

In conclusion, these changes in the Spectroquant® test kits (or future test kits with the same chemistry) produce an identical or equivalent result when compared to the colorimetric nitrate methods listed in 40 CFR part 136 .

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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 4,6
% RSD or correlation coefficient of calibration regression.	Yes, Tables 6,7
Performance range tested with units.	Yes, Tables 4,6,7
Sample(s) used in initial demonstration have the recommended preservative, where applicable.	Yes
Sample(s) used in initial demonstration met recommended holding times, where applicable.	Yes
Interferences.	None, MS/MDS Tables 13 and 14
Document the qualitative identification criteria used.	LFB percent recovery from 3rd party QC samples. Table 8,9. % RSD from 3rd party QC samples. Table 10. MDL determination and comparison to MRL of Spectroquant® test kits. Table 15. MS/MSD . Tables 13 and 14
Performance evaluation studies performed for analytes of interest, where available.	See Tables 6,7, 8, 9, 10, 11, 12, 13, 14, 15
Latest study sponsor or title	NA
Latest study number.	NA
Analysis of external reference material	See Table 8, 9, 10

EPA Method Equivalency Check-Off Table from Richard Reding Memo, *Flexibility to Modify CWA Methods*, 2007	
Equivalency Requirement	**Section in Report**
Results of analyses on reference material from a source different from that used to prepare the calibration standards, if applicable.	See Table 8, 9, 10.
Sources of external reference material, if applicable.	See Table 8
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-NO_3 (E) or report text.
Clean-up procedures.	As per Spectroquant® test kit product flyer or Standard Methods 4500-NO_3 (E) or report text..
Method blank result.	Table 11
Matrix (reagent water, drinking water, effluent)	Final Effluent
Matrix spikes.	Matrix Spike section of the report. See Table 13 and 14
Spiking system, appropriate to the method and application.	Matrix Spike section of the report. See Table 13 and 14
Spike concentrations (with units corresponding to the final sample concentration) and recoveries.	Matrix Spike section of the report. See Table 13 and 14
Source of spiking material.	Muscatine Water Pollution Control Plant
Number of replicate spikes	MS and MSD analyzed and Spectroquant® LFBs. Each sample analyzed spectroscopically and an average

EPA Method Equivalency Check-Off Table from Richard Reding Memo, *Flexibility to Modify CWA Methods*, 2007	
Equivalency Requirement	**Section in Report**
	and standard deviation determined for the % RSD. See Table 9, 10, 13, 14
Initial demonstration of capability.	See Table 12
Precision (analyte by analyte) Duplicates.	See Table 9, 10, 13, 14
Bias (analyte by analyte).	See Table 9, 10, 13, 14
Detection limit (with units; analyte by analyte).	See Table 15
Confirmation of detection limit, if applicable.	See Table 15
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. Table 15 confirms that the Spectroquant® test limits in the product flyer are met.
Qualitative confirmation.	Not Required