Tobacco and Tobacco Products Analytes Sub-Group

Technical Report

Determination of Nitrite and Nitrate in Smokeless Tobacco Products by Ion Chromatography and Continuous Flow Analysis

2016 Collaborative Study

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1. Summary

In 2016, the CORESTA Smokeless Tobacco Sub-Group (now named Tobacco and Tobacco Products Analytes Sub-Group, TTPA) conducted a small scale investigational study involving nine laboratories for the determination of nitrite and nitrate in smokeless tobacco products using ion chromatography (IC) and/or continuous flow analysis (CFA). The goal of this work was to determine if the IC and CFA methods produced similar results for the determination of nitrite and nitrate in smokeless tobacco and provide recommendations to the TTPA for a larger study that would lead to the development of a CORESTA Recommended Method (CRM). Four labs used the supplied IC method while six labs used their in-house CFA methods (one lab performed both IC and CFA studies). The study results demonstrated that the supplied IC method and the participants' CFA methods provided comparable data and are suitable for the determination of nitrite and nitrate in smokeless tobacco. For this reason, the TTPA decided development of a CRM was not necessary.

2. Introduction

At the April 2016 Sub-Group meeting, several members discussed the need for a method for determination of nitrite and nitrate in smokeless tobacco. Although there is a CRM for the determination of nitrate in tobacco and smokeless tobacco products by CFA (CRM No. 36), there is currently not a standard method for the determination of nitrite in these matrices. The objective of this work was to evaluate the supplied IC method and the participants' in-house methods for the determination of nitrite and nitrate in smokeless tobacco. This was intended to be a preliminary study in order to assess the need for the development of a CRM.

In June through August of 2016, nine laboratories participated in the investigational study using either a supplied IC method provided by Altria or their own CFA methods. The CFA methods used by the participating laboratories were very similar to CRM No. 36, except that nitrite was also included. Four laboratories conducted the study using IC and six laboratories conducted the study with CFA (one company used both IC and CFA). Three 2009 CORESTA Reference Products (CRP1, CRP2, and CRP3) and four 2016 CORESTA Reference Products (CRP1.1, CRP2.1, CRP3.1, and CRP4.1) were included in the study.

In the supplied IC method, tobacco samples were extracted with type 1 water and filtered with a syringe filter. Nitrite and nitrate in the filtered extract were separated from the other anions in the sample using ion chromatography and detected using suppressed conductivity.

The CFA methods used by the participants were similar: first, the tobacco samples were extracted with type 1 water. Next, nitrite in the extracts were reacted with sulphanilamide to form the diazo compound and then with N-1-naphthylethylenediamine dihydrochloride to form a coloured complex. The coloured complex was detected with photometric detection. On a second channel of the CFA, the nitrate in the extract was reduced to nitrite with hydrazinium sulphate in the presence of a catalyst. The sample was then taken through the same colorimetric procedures as nitrite. Nitrate was calculated by subtracting the native nitrite from the sum of the reduced nitrate plus the native nitrite.

3. Organisation

3.1 Participants

Nine laboratories participated in the study. A list of the participating laboratories is provided in Table 1. The laboratories are listed in alphabetical order. Four laboratories used the supplied IC methods and six laboratories used their in-house CFA method (one lab used both IC and CFA methods and was assigned 2 different lab codes). The laboratory codes (A-J) used in this report do not correspond to the same order as the list below.

Table 1. List of Participating Laboratories

Laboratory	IC method	In-House CFA method
Altria Client Services LLC, United States	X	
Enthalpy Analytical, Richmond, United States		X
Imperial Tobacco, Hamburg, Germany		X
RAI Services, United States	X	X
Souza Cruz, Brazil		X
Swedish Match, Owensboro, United States	X	
Swedish Match, Sweden		X
Swisher International, Inc, United States		X
University of Kentucky, United States	X	

Note: Not all participants analysed all 7 samples.

3.2 Protocol

Four participants used the IC method that was provided by Altria (Appendix A) while six labs used their in-house CFA method. Three 2009 CORESTA Reference Products (CRPs) and four 2016 CRPs were specified in the study protocol; however, not all laboratories analysed all samples. Each laboratory procured samples from North Carolina State University. Participants were requested to maintain the CRPs at the recommended storage temperature of -20 °C until removal for equilibration to ambient conditions and analysis. Laboratories were requested to report three independent replicates for each sample. The study was conducted in June to August, 2016. The data were reported on a wet weight basis. The sample information is listed in Table 2.

Table 2. Sample Information

Sample ID	Year manufactured	Product type
CRP1	2009	Swedish style snus pouch
CRP2	2009	American-style loose moist snuff
CRP3	2009	American-style loose dry snuff powder
CRP1.1	2016	Swedish style snus pouch
CRP2.1	2016	American-style loose moist snuff
CRP3.1	2016	American-style loose dry snuff powder
CRP4.1	2016	American-style chopped loose-leaf chewing tobacco

4. Results

The raw data for nitrite and nitrate are provided in Tables 3 and 4, respectively. Nine laboratories participated in the study. Not all labs analysed all samples. Laboratory G requested that their data be removed due to methodological differences. The raw data plots are provided in Appendix B.

Table 3. Nitrite in the Study Samples

Lab code		IC, nitrite (µg/g)				CFA, nitrite (µg/g)				
		A	B	C	D	E	F	H	I	J
CRP1	1	ND	1.47	2.48	2.74	<1.0	0.77	1.55	0.49	NA
	2	ND	1.53	2.54	2.60	<1.0	0.82	1.44	0.44	NA
	3	ND	1.56	2.53	2.62	<1.0	0.92	1.31	0.54	NA
CRP2	1	3.45	4.00	5.08	6.02	3.31	4.3	4.83	3.03	NA
	2	3.40	3.75	4.95	6.01	3.15	4.02	4.71	3.01	NA
	3	3.46	4.00	5.15	6.00	2.96	4.08	4.56	3.03	NA
CRP3	1	4.78	9.50	9.37	12.05	8.05	7.55	10.35	9.28	NA
	2	5.06	9.25	9.57	12.13	8.33	7.5	10.31	9.26	NA
	3	4.85	9.25	9.57	12.06	8.30	7.57	10.36	9.33	NA
CRP1.1	1	ND	0.29	1.55	<2.24	ND	NA	0.93	<0.23	NA
	2	ND	0.30	1.46	<2.26	<1.0	NA	0.68	<0.23	NA
	3	ND	0.31	1.41	<2.26	ND	NA	1.10	<0.23	NA
CRP2.1	1	7.42	7.75	8.06	10.12	8.25	NA	9.37	8.49	NA
	2	7.93	7.25	8.45	10.10	8.18	NA	9.21	8.52	NA
	3	7.52	7.25	8.29	10.26	7.98	NA	9.28	8.38	NA
CRP3.1	1	9.06	9.25	13.12	16.81	9.38	NA	12.42	9.41	NA
	2	9.23	9.25	12.35	14.43	9.53	NA	12.75	9.30	NA
	3	8.94	8.50	12.83	14.22	9.94	NA	12.46	9.40	NA
CRP4.1	1	ND	1.50	2.80	<2.12	<1.0	NA	1.01	<0.23	NA
	2	ND	1.50	2.64	<2.12	<1.0	NA	1.09	<0.22	NA
	3	ND	1.75	2.66	<2.13	<1.0	NA	1.02	<0.23	NA

ND indicates the analyte was not detected
The "<" symbol indicates less than the limit of quantitation
NA indicates the laboratory did not analyse the sample

Table 4. Nitrate in the Study Samples

Lab code		IC, nitrate (µg/g)				CFA, nitrate (µg/g)				
		A	B	C	D	E	F	H	I	J
CRP1	1	6607	6159	6451	6140	7136	NA	6728	7314	NA
	2	6731	6420	6667	5959	6891	NA	6871	7396	NA
	3	6542	6885	6587	6066	6925	NA	6886	7181	NA
CRP2	1	15515	15528	14700	13695	14792	NA	15230	15589	NA
	2	15082	15503	14341	13432	15017	NA	15108	15417	NA
	3	15338	15498	14924	13673	16111	NA	15223	15678	NA
CRP3	1	45553	57248	47889	43509	47400	NA	48364	48133	NA
	2	45453	55618	46970	42830	48784	NA	48161	47000	NA
	3	46963	57398	47729	42579	47560	NA	47903	48143	NA
CRP1.1	1	5924	5252	5029	5305	6578	NA	5932	6419	NA
	2	5957	5461	5576	5088	6123	NA	6086	6425	NA
	3	5909	6168	5540	5490	6534	NA	5916	6577	NA
CRP2.1	1	17168	16710	16224	15661	17233	NA	17296	17766	NA
	2	17269	17023	17121	15863	18682	NA	17441	18326	NA
	3	17308	16785	16951	15807	17760	NA	17594	17974	NA
CRP3.1	1	37895	39340	40329	36113	42951	NA	40717	40567	46700
	2	39883	39293	40023	35913	41594	NA	40295	40575	46500
	3	38922	39363	39812	36397	40901	NA	40406	40052	45800
CRP4.1	1	7776	7083	7706	6990	8249	NA	7653	8310	NA
	2	7766	7078	7542	7029	8374	NA	7694	8094	NA
	3	7745	7195	7740	7008	8302	NA	7781	8124	NA

NA indicates the laboratory did not analyse the sample

A formal statistical analysis was not performed due to the small data set; however, descriptive statistics for the two methods are provided in Tables 5 and 6.

The % difference between IC and CFA data were calculated with the formulation below:

$$\%\ \text{difference} = \frac{\text{IC result} - \text{CFA result}}{(\text{IC result} + \text{CFA result})/2} \times 100$$

Table 5. Comparison of Nitrite Determined with IC and CFA

Samples	IC				CFA				%Diff.
	N (Labs)	Average (µg/g)	Stdev (µg/g)	%RSD	N (Labs)	Average (µg/g)	Stdev (µg/g)	%RSD	
CRP1	4	ND - 2.6	--	--	4	<1.0 - 1.4	--	--	--
CRP2	4	4.61	1.05	22.8	4	3.75	0.74	19.7	20.5
CRP3	4	8.95	2.70	30.5	4	8.85	1.11	12.6	1.2
CRP1.1	4	ND - 1.5	--	--	3	ND - 0.9	--	--	--
CRP2.1	4	8.37	1.15	13.7	3	8.63	0.52	6.0	-3.1
CRP3.1	4	11.50	2.80	24.3	3	10.51	1.54	14.6	9.0
CRP4.1	4	ND - 2.7	--	--	3	<0.2 - 1.1	--	--	--

ND = not detected
The "<" symbol indicates less than the limit of quantitation.
ND indicates the analyte was not detected
The "--" symbol indicates standard deviation, %RSD, and % difference could not be calculated

Table 6. Comparison of Nitrate Determined with IC and CFA

Samples	IC				CFA				%Diff.
	N (Labs)	Average (µg/g)	Stdev (µg/g)	%RSD	N (Labs)	Average (µg/g)	Stdev (µg/g)	%RSD	
CRP1	4	6435	291	4.5	3	7036	228	3.2	-8.9
CRP2	4	14769	797	5.4	3	15352	397	2.6	-3.9
CRP3	4	48312	5408	11.2	3	47939	541	1.1	0.8
CRP1.1	4	5558	365	6.6	3	6288	273	4.4	-12.3
CRP2.1	4	16658	607	3.6	3	17786	479	2.7	-6.6
CRP3.1	4	38607	1612	4.2	4	42255	2581	6.1	-9.0
CRP4.1	4	7388	348	4.7	3	8065	282	3.5	-8.8

5. Data Interpretations

This study demonstrates that the supplied IC method (Appendix A) and the CFA methods used by the participants produced comparable data. The nitrite % differences for the methods, for all samples, were ±10 % except for CRP2 which was 20.5 % higher for IC. These results are reasonable considering the small number of participants and the low nitrite levels in the samples. The nitrate % differences for the methods, for all samples, were ±12 % with CFA producing slightly higher results. Again, the data generated with both technologies are reasonable considering the small study size. The limited data set suggests that between-laboratory variability (%RSD) is slightly better for CFA as compared to the supplied IC method and this result is more pronounced for nitrite. Lastly, sensitivity was comparable between the two methodologies. It is worth noting that CFA has considerably higher throughput than IC; however, CFA may be less efficient for a few samples due to the complexity of the instrumentation and reagent preparation. Also, CFA may be less flexible compared to IC due to the analyte specific configuration.

6. Recommendations

The results of this study demonstrate that the supplied IC method shown in Appendix A provides comparable results to the various CFA methods used by the participants and either methodology appears to be fit for the analysis of nitrite and nitrate in smokeless tobacco products. At the last Subgroup meeting held in Berlin, Germany October 9, 2016, the group decided that since IC and CFA appear to be reasonably harmonized there was no need to develop a CRM for the determination of nitrite and nitrate in smokeless tobacco products.

7. Appendices

Appendix A – Ion Chromatography Method

Appendix B – Raw Data plots

Appendix A: Ion Chromatography Method

Determination of Nitrite and Nitrate in Smokeless Tobacco Products by Ion Chromatography

A. Purpose and Scope

1. **Purpose**

   a. The purpose of this method is to quantitatively determine the concentration of nitrite and nitrate in tobacco, cigarette filler, moist smokeless tobacco (MST) and snus using ion chromatography. Results are reported in units of µg/g and may be reported on an as-is (wet) basis.

2. **Scope**

   a. This test method applies to smokeless tobacco samples with concentrations of nitrite in the range of 2-750 µg/g and nitrate in the range of 50-20000 µg/g.

This method was validated using two commercial MST samples and CRP1 and CRP2.

B. Validation

1. The method's repeatability (i.e. within-day) and intermediate precision (i.e. between-day) were evaluated by analyzing CRP2. The repeatability for this method was 7 % RSD for nitrite and 3 % RSD for nitrate. The intermediate precision was 6 % for nitrite and 4 % for nitrate over 3 days.
2. The LOQ is 2µg/g for nitrite and is 50µg/g for nitrate reported on a wet weight basis.
3. The stability of prepared sample extracts was evaluated by analyzing CRP2. Stability was demonstrated for nitrite for up to 48 hours at ambient condition and for up to 7 days when extracts were stored in a refrigerator. Sample extracts should be stored in a refrigerator and, ideally, analysed on an instrument equipped with a temperature control autosampler. If samples are analysed on an instrument without a temperature control autosampler, samples should be analysed within 48 hours. Nitrate is stable in prepared samples for up 7 days at room and refrigerated temperatures.

C. Equipment Requirements, Apparatus and Reagents

1. **Equipment and Apparatus**

   a. Dionex ICS 3000 Ion Chromatograph (IC instrument) with conductivity detector, autosampler, pump, and eluent generator. Or equivalent

   b. Analytical column: IonPac®AS19, 4mm x 250mm, P/N 062885, Thermo Scientific Dionex

   c. Guard column: IonPac®AG19, P/N 062887, Thermo Scientific Dionex

   d. Suppressor AERS 500, 4-mm, P/N 082540, or equivalent, Thermo Scientific Dionex

   e. EGC III Eluent generator cartridge, P/N 074532, Thermo Scientific Dionex

   f. Orbital platform shaker with square platform

D. Chemicals, Reagents, and Supplies

1. Sodium nitrite-ACS Certified grade. Cat#S347-250, Fisher Scientific, or equivalent
2. Sodium nitrate-ACS Certified grade. Cat#S343-500, Fisher Scientific, or equivalent
3. Reagent water (MilliQ water), 18 μΩ or equivalent
4. 1000 ml class A volumetric flask
5. 100 ml class A volumetric flask
6. Bottle-top dispenser, 10-100 ml adjustable, BrandTech Scientific, Fisher Cat# 13-688-232, or equivalent
7. 0.2 μm PVDF syringe filter, 25mm, Whatman, Cat# 09-927-29C, or equivalent

E. Environmental and Sample Requirements

1. **Sample Requirements**

   a. At least 2 grams of tobacco sample per replicate is required for this test method. For regular smokeless tobacco samples such as fine cut, long cut, and snus samples, no further grinding is needed. For pouch products, the pouch should be cut in half and extracted with the tobacco contents.

   b. Tobacco samples stored in the freezer shall be allowed to equilibrate, unopened, in the refrigerator for a minimum of 24 hours. After equilibration in the refrigerator, samples shall be allowed to equilibrate to ambient conditions before being opened for sample preparation.

F. Reagent and Standard Preparation

1. **Reagent Preparations** – Reagent water (MilliQ water) is used for standard preparation and sample extraction.
2. **Standard Preparations**

   a. **Standard Stock Solutions:** Prepare independent standard stock solutions of approximately 100 μg/ml of nitrite and 1000 μg/ml of nitrate. Fill a 1000 ml volumetric flask approximately half full with MilliQ water. Add the approximate weight of neat reagent to the flask that is specified in Table 1. Record the exact weight in order to calculate the true concentration. Mix thoroughly to dissolve the reagent and then make to volume with MilliQ water. The stock and working standards are stable for one year.

Table 1. Standard Stock Solution

Chemical	Purity	Approximate Weight, g	Formula Weight	F.W as Anions	Flask Volume (ml)	Anion Conc, μg/ml
Sodium Nitrite	99.5%	0.1508	69.00	46.00	1000	100
Sodium Nitrate	99.2%	1.3820	84.99	62.00	1000	1000

Example Calculation:

$$\text{Standard Conc} = \text{Wt} * \left(\frac{\text{Formula weight - as anion}}{\text{F.W}} \right) * \left(\frac{1}{\text{Vol}} \right) * \text{purity}$$

Where:

Wt = chemical weight (g)
Formula weight (as anion) = formula weight of anion of chemical used for standard
Vol = final volume (ml)
Purity = purity of the chemical

b. **Calibration Standards:** Using appropriately sized class A volumetric pipettes or mechanical pipettes, add the amount of stock solutions specified in Table 2 to 100 ml volumetric flasks. Add MilliQ water to mark and mix well.

Table 2. Calibration Standards

	Flask Volume (ml)	Nitrite Stock Solution	Nitrate Stock Solution	Nitrite Concentration (µg/ml)	Nitrate Concentration (µg/ml)
Cal 1	100	40 µl	100 µl	0.040	1.00
Cal 2	100	200 µl	500 µl	0.200	5.00
Cal 3	100	1.00 ml	2.00 ml	1.00	20.0
Cal 4	100	5.00 ml	10.00 ml	5.00	100
Cal 5	100	10.00 ml	25.00 ml	10.00	250
Cal 6	100	15.00 ml	40.00 ml	15.00	400

3. **Calibration Check Standard (CCS) Preparations:**

a. **Check standard stock solutions:** Independent stock solutions shall be prepared for the check standards (the calibration stocks should not be used). Prepare check standard stock solutions of approximately 100 µg/ml of nitrite and 1000 µg/ml of nitrate. Procedures and calculations are the same as described in "Standard Stock Preparations".

b. **Check standard:** Using appropriately sized class A volumetric pipettes or mechanical pipettes, add the amount of stock solutions specified in Table 3 to 100 ml volumetric flasks. Add MilliQ water to mark, mix well.

Table 3. Calibration Check Standards

	Flask Volume (ml)	Nitrite Stock Solution	Nitrate Stock Solution	Nitrite Concentration (µg/ml)	Nitrate Concentration (µg/ml)
CCS low	100	500µl	1.00 ml	0.500	10.00
CCS high	100	10.00ml	25.00 ml	10.0	250

G. Instrumentation Settings

1. Set up the Ion Chromatograph, data station, and autosampler according to the manufacturer's instructions. Determination of nitrite and nitrate is achieved using a suppressed conductivity detector using potassium hydroxide eluent generator cartridge in the recycled mode. The operator may optimize the instrument settings according to the examples given below.
2. Example of Instrument Settings
   a. Flow rate: 1.0 ml/min
   b. Column temperature: 30 ^{o}C
   c. Detector cell temperature: 35 ^{o}C
   d. Injection sample loop: 10µl
   e. Suppressor current: 137mA
   f. Flush volume: 500µl
   g. Run time: 30min
   h. Gradient profile for eluent generator is listed in the table below

Table 4. IC Gradient Profile

Time (min)	Conc. of KOH (mM)	Eluent Generator Curve	Flow rate (ml/min)
0	10	5	1.0
12	10	5	1.0
25	55	5	1.0
26	10	5	1.0
30	10	5	1.0

H. Calibration

1. Prior to calibration, any necessary IC system maintenance should be performed and the system should be allowed to equilibrate. System operational suitability should also be assessed. See the Quality Control section for more information about assessing the system suitability.
   a. Generate a new calibration curve before each analytical sequence using fresh aliquots of nitrite and nitrate working standards, Cal 1 through Cal 6.
   b. Generation of Calibration Curves:
      i. When analyzing the calibration standards, the individual analyte concentrations are to be entered into appropriate fields in the quantification method. "Standard" should be selected for the sample type of each working standard.
      ii. Set up the quantitation method using the following parameters: select external calibration for nitrite based on peak height and nitrate based on peak area; calibration type is linear with 1/X weighing and the y-intercept is not forced to zero (XLOff) for both nitrite and nitrate.

Table 5. Example of Peak Table of Quantitation Method

Peak name	Ret. Time[1] (min)	Window	Standard	Int. Type	Cal Type
Nitrite	12	0.100AG	External	Height	XLOff
Nitrate	17	0.300AG	External	Area	XLOff

[1.] Retention times are estimates and may vary with the system used.

I. Test Procedure

1. Sample Extraction

a. Weigh 2.0 g of tobacco material with an analytical balance into a tarred 125 ml Erlenmeyer flask. Record the exact weight of sample.

b. When analyzing pouched products, select a unit number of pouches that comes closest to the target weight of 2.0 g. The pouch(s) shall be cut in half and the tobacco and pouch material shall be added to the extraction vessel.

c. Add 100.0 ml MilliQ water to the flask using a verified dispenser.

d. Cover the flask with a lid or equivalent.

e. Shake samples on an orbital platform shaker or equivalent device at 225 rpm for at least 30 ± 5 minutes.

f. Filter approximately 1 ml of sample directly into 1.5 ml autosampler vial using a 0.22 µm PVDF syringe filter.

2. Sample injection order

a. Reagent blank/water blank (MilliQ water collected from the dispenser used for sample extraction).

b. System Suitability sample (calibration standard 1, triplicate injections). The results of this injection should be checked for acceptable system suitability criteria before proceeding with batch analysis. (See Quality Control Section of the method for acceptance criteria.)

c. Working standards, Cal 1 through Cal 6

d. Water blank

e. Check standards CCS low

f. Check standards CCS high

g. Water blank

h. Reference monitor that contains nitrate and nitrite. Alternatively, CRP1 may be fortified with 20 ppm nitrite.

i. Reference monitor replicate 2

j. Samples in the batch of approximately 15-30

k. Repeat CCS low and CCS high with batches of approximately 15-30 samples

Evaluation of every standard and sample chromatogram is required to assure proper peak assignment and integration.

J. Quality Control

1. Check the instrument suitability by calculating %RSD of peak height for nitrite and peak area for nitrate for the triplicate injections of Cal 1 at the beginning of each sequence. %RSD of the three injections should be less than 15%.
2. Coefficient of determination (R^2) for calibration curve should be greater than 0.99.
3. % Difference of calibration check standards from the theoretical concentration should be within ±15% for the high check standard and within ±20% for the low check standard.

K. Calculations

1. Data are reported to 2 decimal places for nitrite and as a whole number for nitrate in units of µg/g.
2. The concentration of the target analytes in a sample (µg/g), on an as-is basis, is determined using the slope and intercept obtained from the appropriate calibration curve derived from the instrument software (see equation below). Sample weight and dilution factor should be entered in the sample sequence before processing the data.

$$\text{Sample Conc} = \left(\frac{\text{peak response - Int}}{\text{slope}}\right) * \left(\frac{\text{dilution factor}}{\text{Wt}}\right)$$

Where:

Sample Conc (wet) = the calculated concentration (µg/g)
Int = the y-intercept from the calibration curve
Slope = the slope from the calibration curve
Dilution factor = the final volume of the extraction solution, ml
Wt = the sample weight, g

L. Example Chromatograms

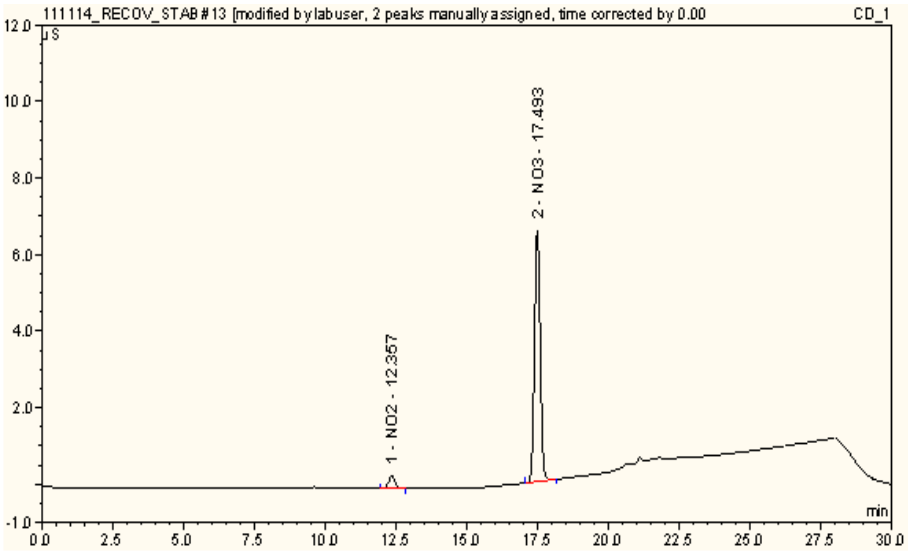


Figure 1: Chromatogram of a calibration standard

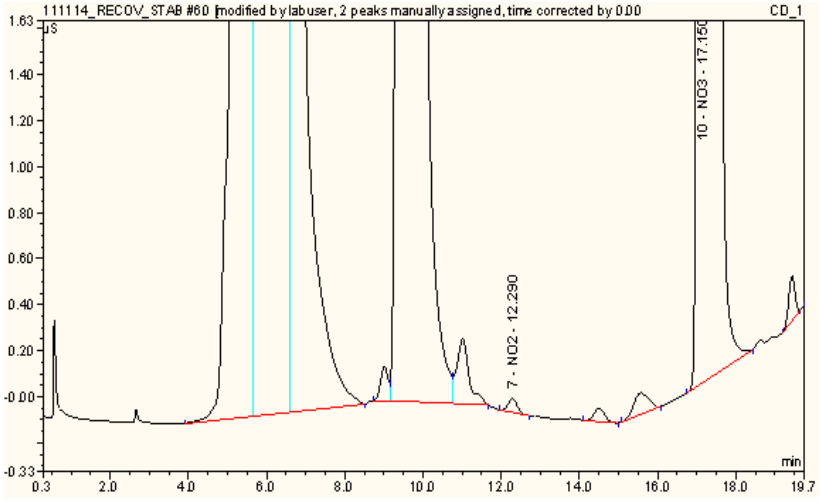
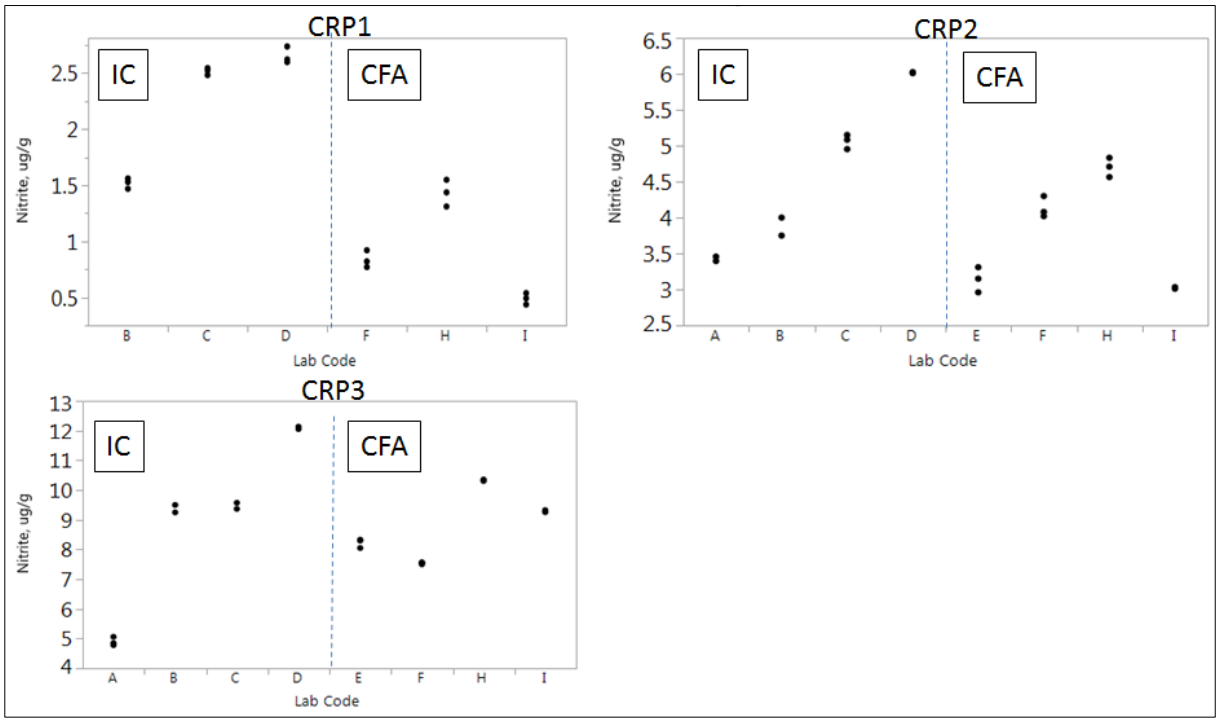


Figure 2: Chromatogram of a MST Sample with Fortified 10µg/g Nitrite

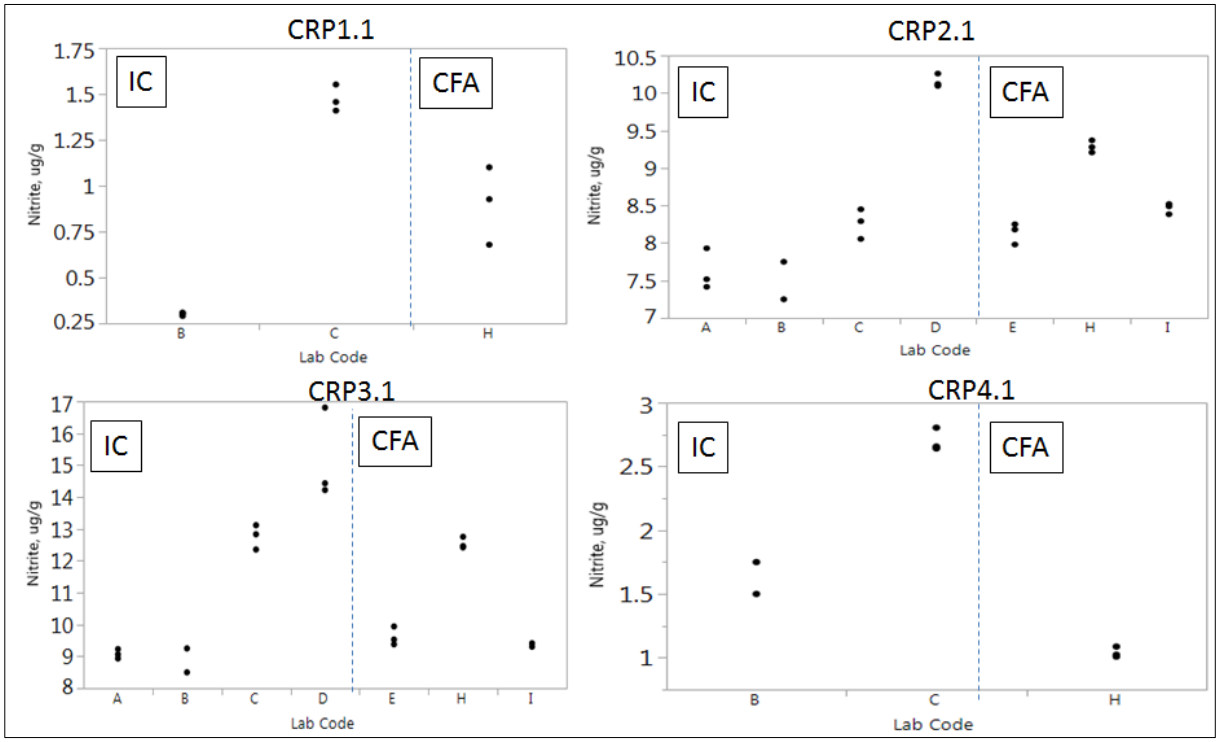
Appendix B: Raw Data Plots

Note: Each plot only includes the labs which analysed samples and excludes the outlier.

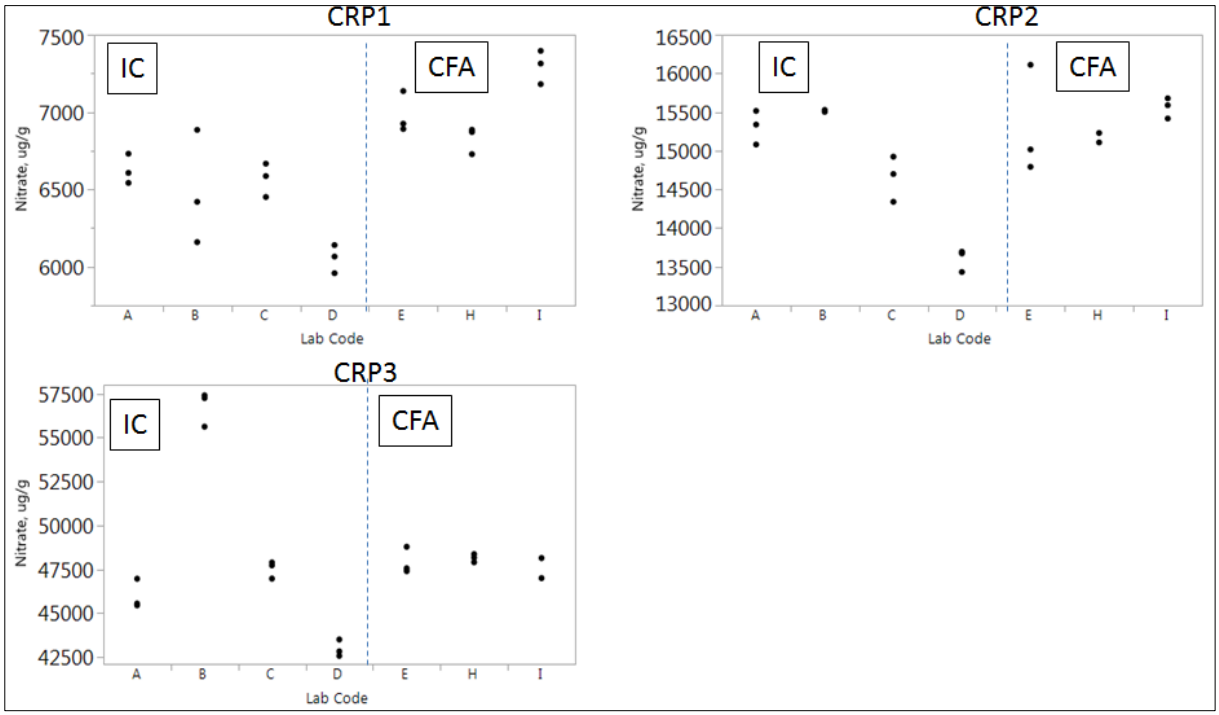
Nitrite in 2009 CRPs



Nitrite in 2016 CRPs



Nitrate in 2009 CRPs



Nitrate in 2016 CRPs