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

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

A. SCOPE

1. This method quantitatively determines the concentration of nitrite and nitrate in moist smokeless tobacco (MST) and snus by ion chromatography (IC). Results are reported in µg/g and may be reported on an as-is (wet) or a dry-weight basis.
2. 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 50-20,000 µg/g.

B. DEFINITIONS

1. **Internal Quality Control Sample (IQCS)** - The IQCS is a method process control sample that is used to assess method performance. Since the IQCS does not contain appreciable levels of nitrite, each replicate of the IQCS is fortified with 50 µg nitrite. The IQCS is run with the routine application of the method and the results charted to verify that the method is in control.
2. **Calibration Check Standard (CCS)** - Standard solution that is prepared separately from the calibration solutions using a different source or lot of material. It is used to verify the standard solution's accuracy and to check the instrument's stability after the calibration and during sample analysis.
3. **%RSD** – Percent relative standard deviation.
4. **Percent Relative Concentration Residuals (%RCR)** - Calculated to show the degree of deviation of individual concentration points from the established calibration regression equation.
5. **Type I water** – ASTM Standard Specification for Reagent Water: D1193 – 06 (2011), with resistivity specified for this method at ≥18.2 MΩ-cm.
6. **Reagent Blank** – A blank that consists of Type I water used to extract the samples.
7. **Extraction Blank** – A blank that consists of the Type I water used to extract the samples taken through the entire sample preparation procedure.
8. **Smokeless Tobacco Products –** For the purposes of this method, all references to smokeless tobacco include both MST and snus product types.

C. RESPONSIBILITIES

1. The designated trained analyst performing the method is responsible for following all steps of the procedure as well as documenting and reporting any procedural deviations from the method to laboratory management.
2. Personnel using this test method are responsible for conducting the analysis in a manner consistent with the safety policies of ALCS.

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D. VALIDATION

1. This method was validated using two commercial MST samples and two CORESTA Reference Products (CRPs). The commercial products included Copenhagen Snuff and Skoal Long Cut Wintergreen. The CRPs included CRP1 (snus-style tobacco product) and CRP2 (MST-style tobacco product).
2. The method's repeatability (i.e., within-day or intra-assay precision) and intermediate precision (i.e., between-day or inter-assay precision) were evaluated by analyzing reference product CRP2.
3. The limit of detection (LOD) and limit of quantitation (LOQ) were determined using the method's lowest calibration standard. The values are reported on a wet (as-is) basis.

   Table 1 provides a summary of the method's precision estimates (R and r) and mean of the results (level) as well as the method LOD and LOQ.

Table 1. Pooled Precision Estimates, LOD, and LOQ

Analyte	Repeatability (r) (as %RSD at defined level)	Intermediate Precision (R) (as %RSD at defined level)	LOD (µg/mL)	LOQ (µg/mL)
Nitrite	7.3% at 3.21 µg/mL	7.3%* at 3.26 µg/mL	0.5	2
Nitrate	3.0% at 13300 µg/mL	4.5% at 14100 µg/mL	0.3	1

* During validation, the intermediate precision was determined to be 6.1% which was below the repeatability value. As a result, the intermediate precision estimate is set to the repeatability value.

4. The stability of prepared sample extracts was evaluated by analyzing CRP2. Stability was demonstrated for nitrite for up to 48 hours at ambient conditions and for up to 7 days when extracts were stored in a refrigerator.

E. EQUIPMENT AND APPARATUS

1. Equipment and Apparatus Required

   Note: If necessary, equivalent items may be used in place of the equipment specified below, with prior authorization from lab management.

   a. Ion Chromatography System, to include:

      Note: Thermo Fisher Scientific Corporation is now the manufacturing company for the Dionex ICS 3000 ion chromatography system listed in this method.

      1) ICS 3000 Ion Chromatography System; Dionex Corp., Sunnyvale, CA, or equivalent.

2) ICS 3000 Dual Pump (Model DP-1) with PEEK flow path, part # 063945/16; Dionex Corp.; Sunnyvale, CA.
3) ICS 3000 DC-1 Detector/Chromatography Module, part # 061767; Dionex Corp.; Sunnyvale, CA.
4) Conductivity Detector CD, part # 061830; Dionex Corp.; Sunnyvale, CA.
5) Conductivity Suppressor ASRS 300, 4 mm, part # 064554; Dionex Corp., Sunnyvale, CA.
6) Eluent Generator Unit, Model EG-1, part # 062256-01; Dionex Corp., Sunnyvale, CA.
7) Eluent Generator Cartridge, potassium hydroxide; part # 074532; Dionex Corp., Sunnyvale, CA.
8) IonPac® AS19, 4-mm x 250-mm analytical column, part # 062885; Dionex Corp., Sunnyvale, CA.
9) IonPac®AG19, 4-mm x 50-mm guard column, part # 062887; Dionex Corp., Sunnyvale, CA
10) ICS 3000 Autosampler; Dionex Corp., Sunnyvale, CA.
11) Dionex Chromeleon Chromatography Software, version 6.8 or above.

b. Analytical Balance, readability 0.0001.
c. Platform Shaker, for 170-mL plastic flasks, capable of 225 rpm or equivalent.
d. Simport™ Scientific Drosophila Stock Polypropylene Bottles, 170-mL, part # 11-888; and caps, part # 11-888-1; available from stockroom or Thermo Fisher Scientific, or equivalent.
e. Fisherbrand™ Autoclavable Bottle-top Dispenser, 10-100 mL, part # 13-615-15; available from Thermo Fisher Scientific Corp., or equivalent.
f. Polycarbonate or Polypropylene Syringes, 3-mL, BD brand with Luer-Lok™ tips, part # 14-823-435; available from Thermo Fisher Scientific.
g. Syringe filters, 25-mm, 0.2-µm Polyvinylidine Fluoride (PVDF) w/ glass membrane filter (GMF), part # 6872-2502; available from Whatman, or equivalent.
h. Autosampler vials and caps (vial kit), 1.5-mL vial w/ split septum (teflon-backed silicone), part # 055427; Dionex Corp., Sunnyvale, CA.
i. Eppendorf™ Repeater™ M4 Manual Hand-held Pipette Dispenser, part # 14-287-150; available from Thermo Fisher Scientific.

2. Instrument Setup (See Appendix: Attachment 2 for Example Instrument Program)

   a. Chromatographic Parameters:

Table 2. Chromatographic Settings

Parameter	Setting
Eluent A	Type I water
Eluent B	EG Cartridge w/ conc. potassium hydroxide (KOH)
Column Temperature	30 °C
Eluent Rate:	1.00 mL/min
Autosampler Temperature	4 °C
Injection Volume	10 µL
IC Run Time	30 min
Needle Height	2 mm
Syringe Speed	4
Pressure Range	200 psi (min) / 3000 psi (max)
Flush Volume	500 µL
Data Acquisition	0-30 min

   b. Eluent gradient:

Table 3. IC Pump Settings

Time (min)	KOH (mM)	Eluent Curve	Flow Rate (mL/min)	Suppressor Current (mA)
0.0	10	5	1.00	137
12.0	10	5	1.00	137
25.0	55	5	1.00	137
26.0	10	5	1.00	137
30.0	10	5	1.00	137

3. Instrument Maintenance

   Proper maintenance of the IC instrumentation is important for optimum performance of the test method. Refer to the instrument manual for proper maintenance. Set up the Ion Chromatograph, data station, and autosampler

according to the manufacturer's instructions. Suggested operating conditions are as follows:

> Turn on pump, eluent generator, column_TC, compartment_TC, and suppressor; let the instrument warm up and column equilibrate for at least 30 minutes.

F. CHEMICALS AND REAGENTS

1. Chemicals Required
   a. Nitrite reference standard, 100 µg/mL, ISO Guide 34 certified, available from AccuStandard Corp., part # IC-NO2-1X-5, 500-mL, or equivalent ISO Guide 34 certified reference standard solution. For preparation of calibration standards.
   b. Nitrate reference standard, 1000 µg/mL, ISO Guide 34 certified, available from AccuStandard Corp., part # IC-NO3-10X-5, 500-mL, or equivalent ISO Guide 34 certified reference standard solution. For preparation of calibration standards.
   c. Type I water: ≥18.2 MΩ-cm.
   d. Nitrite secondary source standard, 100 µg/mL, from High Purity Standards, part # IC-N, 100-mL. For preparation of CCS.
   e. Nitrate secondary source standard, 1000 µg/mL, from High Purity Standards, part # IC-NO-M, 100-mL. For preparation of CCS.
2. Reagent Preparation
   a. Type I water, resistivity ≥18.2 MΩ-cm, either purchased or from a water purification system – to be used for standard preparation and sample extraction. Use fresh water weekly.
3. Standard Preparation
   a. Calibration Standards
      1) Standard Stock Solutions
         a) Nitrite reference standard (ISO Guide 34 certified), 100 µg/mL; to be used as purchased.
         b) Nitrate reference standard (ISO Guide 34 certified), 1000 µg/mL; to be used as purchased.
      2) Calibration Standards: Using Class-A volumetric pipettes or mechanical pipettes, add the amount of ISO Guide 34 reference standard stock solutions specified in Table 4 to 100-mL Class-A volumetric flasks. Add

Type I water to the mark and mix well. Transfer to 100 mL bottles and store refrigerated at 4°C. The calibration standard solutions expire one year after preparation.

Table 4. Calibration Standards

Calibration Standard ID	Flask Volume (mL)	Nitrite Stock Solution (mL)	Nitrate Stock Solution (mL)	Nitrite Conc. (µg/mL)	Nitrate Conc. (µg/mL)
STD 1	100	0.0400	0.100	0.0400	1.00
STD 2	100	0.200	0.500	0.200	5.00
STD 3	100	1.00	2.00	1.00	20.0
STD 4	100	5.00	10.0	5.00	100
STD 5	100	10.0	25.0	10.0	250
STD 6	100	15.0	40.0	15.0	400

b. Check Standard Solutions (CCS)

1) Stock Solutions

a) Nitrite reference standard (ISO Guide 34 or other certified), 100 µg/mL

b) Nitrate reference standard (ISO Guide 34 or other certified), 1000 µg/mL

2) Add the amount of each stock solution specified in Table 5 to a 100-mL Class-A volumetric flask. Add Type I water to the mark and mix well. Transfer to 100 mL bottles and refrigerate at 4 °C. The check standard solutions expire one year after preparation.

Table 5. Calibration Check Standards

Check Standard ID	Flask Volume (mL)	Nitrite Stock Solution (mL)	Nitrate Stock Solution (mL)	Nitrite Conc. (µg/mL)	Nitrate Conc. (µg/mL)
CCS low	100	0.500	1.00	0.500	10.0
CCS high	100	10.0	25.0	10.0	250

G. SAMPLE REQUIREMENTS

1. Tobacco shall be ground at ambient temperature as described in WI 097-1108 “Sample Preparation”.
2. A minimum of 2 g of tobacco is required for each replicate sample analysis. For smokeless tobacco samples such as fine cut MST, long cut MST, and snus

samples, no further grinding is needed. It is recommended to submit a minimum of 15 g of tobacco for this test method.

3. IQCS samples

   IQCS monitor: a minimum of 1.00 ± 0.025 g per replicate.

H. PROCEDURE

1. Extraction Blank
   a. Dispense 100 mL of Type I water using a bottle-top dispenser or 100-mL, To-Deliver (TD) graduated cylinder into a 170-mL disposable plastic flask and seal with a cap.
   b. Place the flask on the orbital shaker and shake for a minimum of 30 minutes at 225 rpm.
   c. Within one hour after shaking, use a 3-mL syringe to filter the required number of aliquots of the extraction blank through a 0.2-µm PVDF syringe filter into an autosampler vial.
2. Fortified IQCS (3R4F)
   a. Weigh 1.00 ± 0.025 g of the IQCS monitor into a 170-mL disposable plastic flask.
   b. Using a mechanical pipette, add 0.5 mL of the nitrite stock standard (100 µg/mL) to each IQCS sample.
   c. Dispense 100 mL of Type I water using a bottle-top dispenser or TD graduated cylinder and seal with a cap.
   d. Place the flask on the orbital shaker and shake for a minimum of 30 minutes at 225 rpm.
   e. Within one hour after shaking, use a 3-mL syringe to filter an aliquot of the extract through a 0.2-µm PVDF syringe filter into an autosampler vial.
3. Sample Handling
   a. Weigh 2.00 ± 0.05 g of tobacco material into a 170-mL disposable plastic flask.
      1) Pouched smokeless tobacco products: Manually open each pouch using scissors, a knife, or tearing by hand. Use nitrile gloves when handling pouches. Transfer the tobacco material into a sample bottle to make a composite sample and discard the pouch material. Bottle a minimum of 15 grams; the composite tobacco sample may be used for other tests in

addition to Nitrite and Nitrate. Mix the composite sample by shaking or stirring before removing sample aliquots.

b. Dispense 100 mL of Type I water using a bottle-top Dispenser or graduated cylinder and seal with a cap.

c. Place the flask on the orbital shaker and shake for a minimum of 30 minutes at 225 rpm.

d. Within one hour after shaking, use a 3-mL syringe to filter an aliquot of the extract through a 0.2-µm PVDF syringe filter into an autosampler vial.

4. Calibration

a. Prior to performing a calibration, perform any necessary IC system maintenance and assess the system operational suitability as described in the Quality Control and Acceptance Criteria section.

b. Acquire a new calibration curve daily before performing sample analysis. Use fresh aliquots of working standards, STD 1 through STD 6. Transfer an aliquot from each working standard into an autosampler vial. Place the standard vials in the autosampler. Inject 10 µL of each standard onto the ion chromatograph.

c. Generation of Calibration Curves:

1) Consult the appropriate Chromeleon manuals for system software calibration information. The Dionex Chromeleon software is used to construct a calibration curve by plotting concentration (µg/mL) of the standard versus the peak response for each of the working standards as shown in Table 6.

Note: Based on the sample's actual weight, extraction volume, and dilution factor, concentration in µg/mL is converted into µg/g by the Chromeleon quantitation method (.qnt file).

2) When analyzing the working standards, the analyte actual concentrations of the standards are to be entered into the appropriate quantitative method (.qnt file). "Standard" should be selected for the sample type of each working standard.

3) Set up the quantitation method using the following recommended 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 6. Peak Table of Quantitation Method

Peak name	Ret. Time (min)	Window (min)	Standard	Integration Type	Calibration Type
Nitrite	12.3	0.100 min AG*	External	Height	XLOff
Nitrate	17.4	0.300 min AG*	External	Area	XLOff

*AG: "Absolute/Greatest" or largest peak within the target window

Note: Retention times may decrease with extended column use. If necessary, adjust retention times in the quantitation method based on changes to standard retention times.

5. Analysis

   a. The Analytical Sequence (normally created in LIMS)

      Analyze the calibration standards (from low to high concentration) and tobacco samples including reagent blanks, calibration check standards and control samples. An example sequence is shown below:

      Reagent blank
      System suitability check (STD 1) in triplicate
      Std 1
      Std 2
      Std 3
      Std 4
      Std 5
      Std 6
      CCS Low
      CCS High
      IQCS
      Extraction blank
      ~15-30 samples
      CCS Low
      CCS High
      IQCS
      Extraction blank
      ~15-30 samples
      Extraction blank
      IQCS
      CCS Low
      CCS High
      Wash

Note: There is no requirement for the number CCSs to be analyzed in a batch except that all samples must be bracketed by passing CCSs. The analyst should determine if it is appropriate to add CCSs in the middle of a sample batch based upon batch size and the length of a run.

6. Calculations and Reporting

   a. Report the amount of analyte in µg/g, as determined by the following calculation:

$$\text{Analyte (µg/g)} = \frac{C \times V \times DF}{W}$$

Where:

C = Analyte concentration obtained from the regression calibration curve (µg/mL)

V = Extraction volume (mL)

DF (Dilution Factor) = Final volume of diluted extract (mL)/extract aliquot volume (mL)

W = Sample weight (g)

   1) Example calculation with no dilution:

$$\frac{1.5135\ \mu g}{mL} \times \frac{100\ mL}{2.0112\ g} \times 1 = 75.3\ \mu g/g$$

   2) Example calculation with dilution:

      1.00 mL aliquot of extract, diluted to a final volume of 10.0 mL:

$$\frac{1.6251\ \mu g}{mL} \times \frac{100\ mL}{2.0165\ g} \times 10 = 806\ \mu g/g$$

7. Quality Control and Acceptance Criteria

   a. System Suitability Evaluation

      System suitability should be evaluated prior to calibration using the criteria below:

      1) Detector drift: Equilibrate the ion chromatograph by flowing eluent through the column at 1.0 mL/min before the first injection. Ensure the conductivity detector reading is stabilized to within ±0.001 µS for at least 1 minute. If drift is greater than 0.001 µS, check that the system pressure is

stable, equilibrate the system longer, and/or change the Type 1 water eluent.

2) Extraction Blank (EB) and Reagent Blank (RB): Evaluate the RB and EBs for the presence of background interferences indicating system contamination or instrument malfunction. If there is a detectable quantity of analyte in the blank greater than ½ the STD 1 peak response, evaluate the source of contamination and take appropriate action.

3) Check the instrument suitability by calculating %RSD of peak height for nitrite and peak area for nitrate using triplicate injections of STD 1 at the beginning of each sequence. %RSD should be less than 15% based on the average of the three injections.

4) If there is a shift in peak retention times, check that the system pressure is stable, equilibrate the system longer, and/or change the Type 1 water eluent. Recalibrate the system.

b. Calibration:

1) Consider the calibration curve valid if the following conditions are met:

a) Coefficient of determination (R^2) values for nitrite and nitrate ≥ 0.990.

b) %RCR value of the individual calibration points:

(1) STD 1 ≤ 20%, and

(2) STDs 2-6 ≤ 15%.

2) If the R^2 value for the calibration is less than 0.990, reject the calibration. If an identifiable cause can be found to reject one of the standards from the calibration curve (e.g., a standard vialed twice) then one standard may be eliminated from the calibration. The results of the sequence must then be reprocessed and a new calibration curve generated. If the R^2 value for the new calibration curve is 0.990 or higher, the calibration is accepted. If, upon recalibration, the R^2 value for the calibration is still less than 0.990, the calibration is rejected. The sequence must be run again using new set of standards.

3) The first or last point of the calibration curve may be dropped due to failing %RCR as long as <u>all</u> samples fall within the remaining calibration standards.

c. Chromatogram Evaluation: Evaluation of every standard and sample chromatogram after analysis is required to assure proper peak assignments and integration. Check and correct integration to reflect accurate peak

baselines. Example chromatograms of Calibration Standard 1 and a sample are included in the Appendix (Attachment 1 – Figures 1 and 2).

d. Extraction Blank (EB): Analyze the EB to evaluate the presence of background interferences and to detect instrument malfunction. Analyze at least one EB sample with each sample batch. If there is a detectable quantity of analyte in the EB greater than ½ the STD 1 peak response), evaluate the source of contamination and take appropriate action (which may include discarding the batch).

e. Calibration Check Standard (CCS): The validity of the calibration curves must be checked after calibration and during an analysis of the batch by injecting the CCS. All samples must be bracketed by passing CCSs. The %RCR for the CCS should be within ±15% of the nominal concentration for the analyte. The CCS results that do not pass the acceptance criteria suggest that the calibration curves are no longer valid. The samples analyzed after the last passing CCS must be reanalyzed with a new calibration unless there is an assignable cause.

f. Internal Quality Control Sample (IQCS):

1) Use the designated tobacco material to assess method performance and to verify the method is in control.

2) Analyze three (3) IQCS samples for each batch. The recommended practice is to analyze the control samples: 1) following the first CCS, 2) in the middle of the run, and 3) preceding the last CCS.

3) Confirm that all IQCS results are within established control charts limits. This sample should be fortified with 0.5 mL of 100 µg/mL of nitrite (50 µg) and recovery should be within 15%.

4) Evaluate the quality control results for deviation against the defined recovery criteria (see above). Document and review any deviations with laboratory management as necessary.

I. REFERENCES

1. Validation Report, “Determination of Nitrite and Nitrate in Smokeless Tobacco by Ion Chromatography”, Dec. 5, 2011.
2. WI 097-1108, “Sample Preparation”.
3. Instrument manual, Chromeleon software v.6.8 SR11.

J. FORMS

1. 099-5128 Standards Preparation template.

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K. APPENDIX

Attachment 1: Example Chromatograms

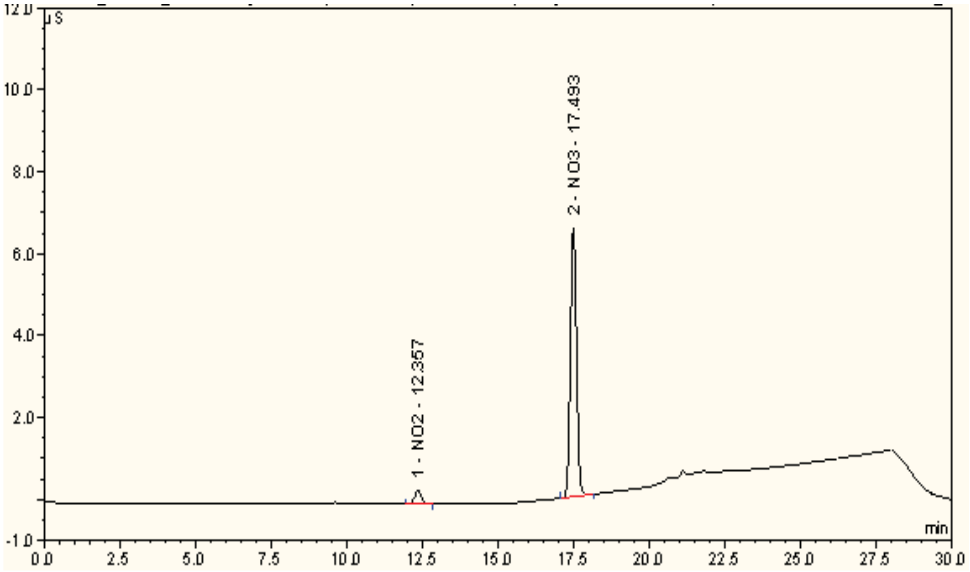

Figure1. Chromatogram of a Standard Cal3

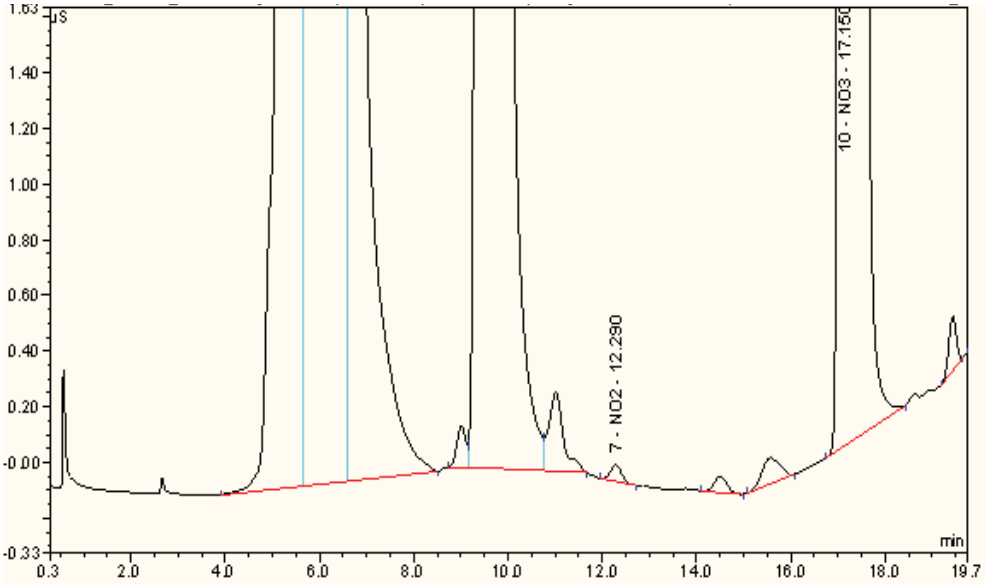

Figure 2. Chromatogram of a Nitrite-Fortified MST Sample

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Attachment 2: Example Chromeleon Instrument Program

```
Sampler.AcquireExclusiveAccess
                Flush                                   Volume = 500
    Wait        FlushState
    Column_TC.AcquireExclusiveAccess
    Compartment_TC.AcquireExclusiveAccess
    Pressure.LowerLimit = 200 [psi]
    Pressure.UpperLimit = 3000 [psi]
    MaximumFlowRamp = 6.00 [ml/min²]
    %A.Equate =                                         "%A"
    %B.Equate =                                         "%B"
    %C.Equate =                                         "%C"
    %D.Equate =                                         "%D"
    CR_TC =     On
    NeedleHeight = 2 [mm]
    CutSegmentVolume = 10 [µl]
    SyringeSpeed = 4
    TrayTemperature = 5 [°C]
    CycleTime =                                         0 [min]
    WaitForTemperature = False
                Pump_1_Pressure.Step = 0.20 [s]
                Pump_1_Pressure.Average = Off
                Data_Collection_Rate = 5.0 [Hz]
                Temperature_Compensation = 1.7 [%/°C]
                CellHeater.Mode = On
                CellHeater.TemperatureSet = 35.00 [°C]
                Column_TC.Mode = On
                Column_TC.TemperatureSet = 23.00 [°C]
                Compartment_TC.Mode = On
                Compartment_TC.TemperatureSet = 30.00 [°C]
                Suppressor1.Type = ASRS_4mm
                CurrentSet = 137 [mA]
                Flow =                                  1.000 [ml/min]
                %B =                                    0.0 [%]
                %C =                                    0.0 [%]
                %D =                                    0.0 [%]
                Pump_1.Curve = 5
                Wait  Column_TC.TemperatureState
                Wait Compartment_TC.TemperatureState
                Suppressor1.Carbonate = 0.0
                Suppressor1.Bicarbonate = 0.0
                ; Suppressor1.Hydroxide = 55.0
                ; Suppressor1.Tetraborate = 0.0
                ; Suppressor1.Other eluent = 0.0
                ; Suppressor1.Recommended Current = 137
                Wait                                    SampleReady

                0.000                                   CDet1.Autozero
                Concentration = 10.00 [mM]
                EGC_1.Curve = 5
    Load
                Wait                                    CycleTimeState
                Inject
                Wait                                    InjectState
```

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	Pump_1_Pressure.AcqOn CD_1.AcqOn CD_1_Total.AcqOn Sampler.ReleaseExclusiveAccess Concentration = 10.00 [mM] EGC_1.Curve = 5	
	12.000 EGC_1.Curve = 5	Concentration = 10.00 [mM]
	25.000 EGC_1.Curve = 5	Concentration = 55.00 [mM]
26.000	Concentration = 10.00 [mM] EGC_1.Curve = 5	
30.000		
	Pump_1_Pressure.AcqOff Concentration = 10.00 [mM] EGC_1.Curve = 5	
	CD_1.AcqOff CD_1_Total.AcqOff Compartment_TC.ReleaseExclusiveAccess Column_TC.ReleaseExclusiveAccess End	