

# Development of a Cigarette Tobacco Filler Standard Reference Material

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### **Details concerning sample preparation (nicotine and nitrosamines).**

Different approaches were used to process samples for the determination of nicotine and nitrosamines to achieve independence in the analytical procedures. For all methods, samples were selected across the production lot using a stratified random sampling scheme. Specific details are compiled in Table 1 and below.

**PREP1.** Approximately 1 gram of tobacco was removed from an individual jar and ground to a fine powder using a Retsch RM 100 automated mortar and pestle (Haan, Germany). Approximately 0.25 g of the ground material and the internal standard were combined in 15 mL polypropylene tubes (exact masses known). Aqueous ammonium acetate (100 mmol/L, 5 mL) was added to each tube, vortex mixed for a few seconds, then placed on a vortex mixer for 60 min to perform the extraction. After the extraction period, the samples were filtered using 0.45  $\mu$ m PVDF membrane syringe filters and the filtrate was transferred into autosampler vials for analysis. The ammonium acetate extraction solution was analyzed as a blank sample to ensure there were no interference peaks or sample carry over between analytical runs.

**PREP2a.** The entire contents of a jar were ground to a fine powder using a Retsch RM 100 automated mortar and pestle mill. Approximately 1 g subsamples of ground tobacco, and aliquots of the internal standard solutions were weighed into 15 mL polypropylene centrifuge tubes by difference. Methanol aliquots (5 mL) were added to each sample for extraction. The samples were extracted by rotational inversion mixing at 6.3 rad/s (60 rpm), for 18 h. After extraction, the slurries were centrifuged for 10 min at 393 rad/s (3750 rpm; 3210 g). Unfiltered aliquots of the supernatant solution were placed in 2-mL autosampler vials for analysis.

**PREP2b.** The same procedure for PREP 2a was used, except for 100 mmol/L aqueous ammonium acetate as the extraction solvent.

**PREP3.** Samples were processed as in PREP 2a, except for 7.5 mL of aqueous sodium hydroxide (2 N) as the extraction solvent. The samples were extracted by rotational inversion mixing at 6.3 rad/s, for 15 h. After extraction, the slurries were centrifuged for 15 min at 393 rad/s. Next, 1 mL aliquots of the supernatant solution were neutralized using 120  $\mu$ L glacial acetic acid. A precipitate formed, which was removed by centrifugation for 15 min at 393 rad/s. The supernatant solution was placed in 2 mL autosampler vials for analysis.

**PREP4 (CDC).** Subsamples (400 mg  $\pm$  5 mg) of tobacco were mixed with 2 mol/L sodium hydroxide (1 mL) for 15 min. Methyl *t*-butyl ether containing the internal standard was added, and the slurry was extracted by rotational inversion mixing at 7.3 rad/s (70 rpm) for 1 hour. The organic layer was separated and analyzed by gas chromatography with mass spectrometry (GC-MS).

**PREP5.** Samples of 1 g each were weighed from a single container of the material, and each aliquot was ground separately using a Retsch RM 100 automated mortar and pestle mill. The ground aliquots and the internal standard solutions were weighed into 15-mL polypropylene centrifuge tubes by difference. Five milliliter aliquots of 100 mmol/L aqueous ammonium acetate were added to each sample for extraction. The samples were extracted by rotational inversion mixing at 6.3 rad/s, over a period of 14 h. After

extraction, the slurries were centrifuged for 10 min at 393 rad/s. Unfiltered aliquots of the supernatant solution were placed in 2-mL autosampler vials for analysis.

#### **Details concerning sample preparation (volatiles/moisture).**

Different approaches were also used to characterize samples for volatile components that included oven drying under different conditions, desiccator drying to constant mass, and Karl Fischer determination of water. The differences in methodology are summarized in Table 1.

**DRY1.** The sample jars were gently mixed prior to sampling. An aliquot ( $\approx 0.5$  g) was removed from each container of candidate SRM 3222 and placed in pre-weighed, glass weighing vessels to an approximate depth of 2 cm. The vessels were again weighed and placed in a desiccator over magnesium perchlorate ( $\text{Mg}(\text{ClO}_4)_2$ ). The samples were removed after 7 d and the weights were recorded. The samples were placed back in the desiccator, weighed and the weights recorded on the following days: day 14, day 21, day 28, and day 35. This approach assumes that all mass losses were due to loss of moisture alone.

**DRY2.** Samples ( $\approx 0.5$  g) were placed in pre-weighed, glass weighing vessels to an approximate depth of 2 cm. The vessels were weighed and placed in a forced air oven set at 80 °C. After 3 h, the samples were removed and weighed. As with DRY1, this approach assumes that all mass losses were due to loss of moisture alone.

**DRY3.** The previous procedure (“DRY2”) was used at 100 °C.

**DRY4 (commercial lab).** Sample aliquots (10 g) were weighed into sample tins and placed into a Hearson Tobacco Oven for 16 hours. After drying, the samples were removed and placed in a desiccator for at least 15 min or until cool. The dry mass was determined, and the oven volatiles calculated by difference.

**DRY5.** Samples were stored at room temperature until run on the Karl Fischer system. The analysis of water in tobacco was made on a coulometric Karl Fischer system with an attached oven. The coulometric Karl Fischer system was set up using a generator electrode without a diaphragm, using 150 mL Hydranal Coulomat AG in the Karl Fischer vessel as the working medium. The Karl Fisher system was started and allowed to run overnight to fully equilibrate.

On the day of the measurements, an initial baseline drift was determined by performing a titration with the system as-is for a few minutes. This was followed by two to four validation runs using 40 mg (nominal) of water-saturated octanol (WSO) and/or gravimetrically prepared water in octanol standard to ensure the instrument was operating within specifications. The standards were injected into the Karl Fischer vessel through a silicone septum via a gas-tight syringe. The amount of water standards injected into the Karl Fischer cell was determined by weighing the injection syringe before and after the injection on an analytical balance.

After the validation runs, the sample vials were inserted into the Karl Fischer oven that was maintained at a constant temperature high enough to release the moisture from the samples. The temperatures used were

between 85 °C and 100 °C, based on data from the other loss on drying methods used for tobacco. The titration was started, and then the septum cap was pierced with the oven's needle to start the transfer of evolved water vapor to the Karl Fischer cell. The water vapor was transferred into the Karl Fischer titration cell using dry nitrogen as the carrier gas, flowing at a rate of 40 ml/min. The transfer is made possible by piercing the septum of the headspace vials with a needle assembly that allows the carrier gas to flow in from the supply and out to the Karl Fischer cell. The stirring rate in the Karl Fischer cell was previously set high enough so that the residence time of the gas bubbles in the solution was between 10 and 20 seconds to help maximize the uptake of the water vapor into the solvent in the Karl Fischer cell.

At the completion of a titration, the needle was withdrawn from the vial, and cleaned of any residual sample. The old sample vial was removed from the oven and a new sample was placed in the oven for the next titration. After allowing the sample to come to room temperature, the sample vials were weighed a second time to determine the total mass lost in the oven heating experiment.

All titrations were run for a set length of time rather than by electrochemical potential of the cell alone (generally 30 min for the validation runs and 90 min for the samples run in the oven). The drift of the instrument was calculated at the conclusion of every run over two successive 10 min intervals to check for consistency in the baseline and to correct the final Karl Fischer signal due to the system drift. Calibration was accomplished with a series of calibration solutions prepared gravimetrically by the addition of water into anhydrous 1-octanol <sup>1</sup>.

**DRY6.** Moisture was determined as mass loss upon heating. Aliquots of 1.5 g were dried at 99 °C ± 1.0 °C for 3 h, and the moisture content is expressed as the difference of sample masses before and after drying.

#### **Details concerning chromatographic methods.**

Several chromatographic methods were used with different columns and conditions to evaluate potential interferences that might result in biases. The gradients were developed to include nicotine and the tobacco specific nitrosamines. Separation examples are provided in Figures S3 – S8 for extracts of SRM 3222.

**LC1.** For analyses performed with the ACE 3 C18 column (4.6 x 150 mm; 3 µm particle size), a flow rate of 0.5 mL/min was used and column temperature was held at 30 °C. The mobile phase consisted of (A) 100 mmol/L ammonium acetate in water pH 5.0 and (B) acetonitrile (ACN). Initial conditions 95:5 A:B; hold for 2 min; linear gradient to 100% B in 3 min, and hold for 3 min; linear gradient to initial conditions over 0.5 min and hold for 1.5 min.

**LC2.** An XTerra MS C18 column was used (4.6 x 50 mm; 5 µm particle size) to achieve method independence. The flow rate was 0.2 mL/min and column temperature was held at 50 °C. The mobile phase consisted of (A) 5 mmol/L ammonium acetate in water and (B) 5 mmol/L ammonium acetate in acetonitrile. The following chromatographic conditions were employed. Initial conditions 60:40 A:B; hold for 1 min; linear gradient to 30:70 A:B in 6 min, linear gradient to 100% B in 0.5 min and hold for 1 min; linear gradient to initial conditions over 0.5 min and hold for 1.5 min.

**LC3.** For analyses performed with the Ascentis Express F5 pentafluorophenyl column (4.6 x 100 mm; 2.7  $\mu$ m particle size), a flow rate of 0.5 mL/min was used and column temperature was held at 50 °C. The mobile phase consisted of (A) 5 mmol/L ammonium acetate in water and (B) 5 mmol/L ammonium acetate in ACN. Initial conditions 75:25 A:B; hold for 3 min; linear gradient to 100% B in 7 min, and hold for 1 min; linear gradient to initial conditions over 0.5 min and hold for 1.5 min.

**LC4.** A different gradient was also used with the Ascentis Express F5 column. A flow rate of 0.5 mL/min was used and column temperature was held at 50 °C. The mobile phase consisted of (A) 5 mmol/L ammonium acetate in water and (B) 5 mmol/L ammonium acetate in ACN. Initial conditions 95:5 A:B; hold for 1 min; linear gradient to 100% B in 9 min, and hold for 2 min; linear gradient to initial conditions over 0.5 min and hold for 1.5 min.

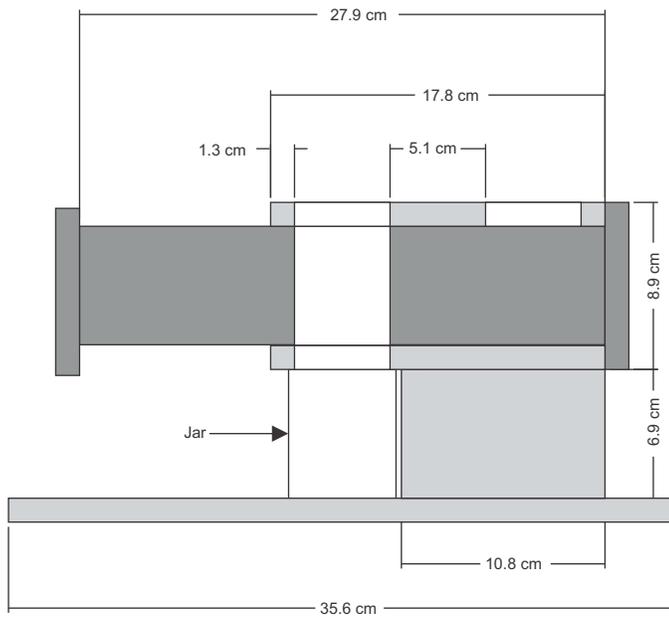
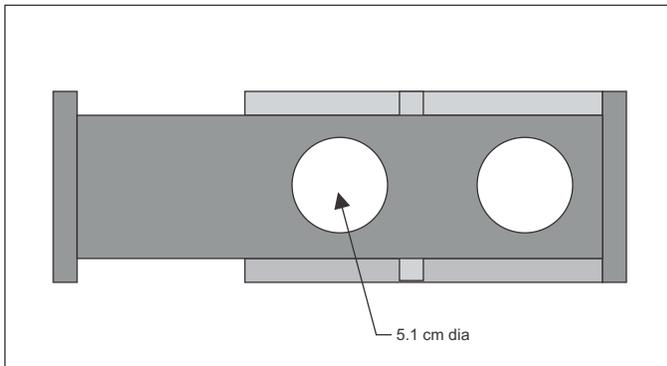
**LC5.** An Xterra MS C18 column was used (4.6 x 50 mm; 5  $\mu$ m particle size) using the same mobile phase conditions as in LC4.

**LC6.** An Ascentis Express F5 pentafluorophenyl column (4.6 x 100 mm; 2.7  $\mu$ m particle size), was used with a flow rate of 0.5 mL/min and the column temperature was held at 30 °C. The mobile phase consisted of (A) 100 mmol/L ammonium acetate in water pH 5.0 and (B) acetonitrile (ACN). Initial conditions 95:5 A:B; hold for 2 min; linear gradient to 100% B in 3 min, and hold for 3 min; linear gradient to initial conditions over 0.5 min and hold for 1.5 min.

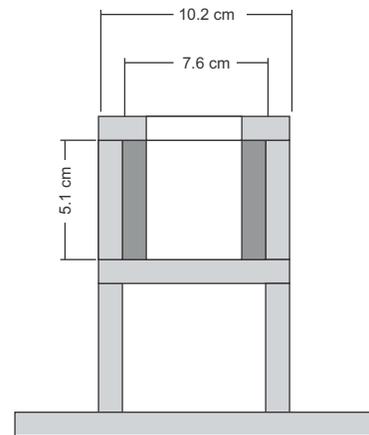
#### Reference List

1. Lang, B. E. *J. Chem. Eng. Data* **2012**, 57 (8), 2221-2226.

## Top View



## Side View



## End View

Figure S1. Top, side and end views of a device for filling jars with the cigarette tobacco filler. The dark grey sliding piston alternately fills and empties a chamber with tobacco into the jar. The hopper for introducing tobacco into the filler is not shown. See Figure S2 for a photographic representation.

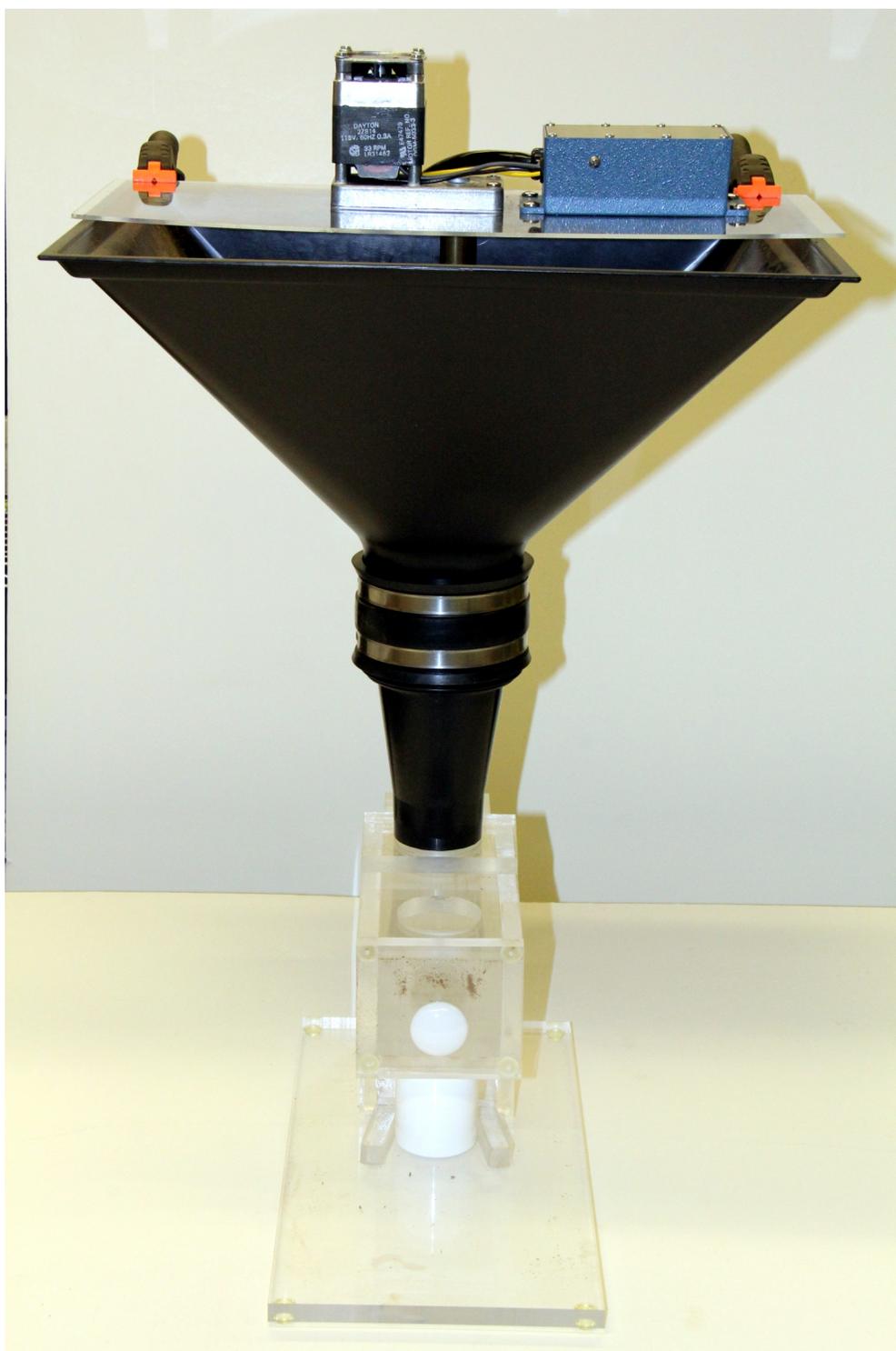


Figure S2. Filling device shown with hopper. A 30 rpm motor drives a shaft with alternating teeth to provide agitation of the tobacco to promote flow into the device. The push-pull action of the device introduces a measured volume of tobacco into each jar. A tamping rod is used to compress the tobacco to facilitate removal of the filled jar from the device.

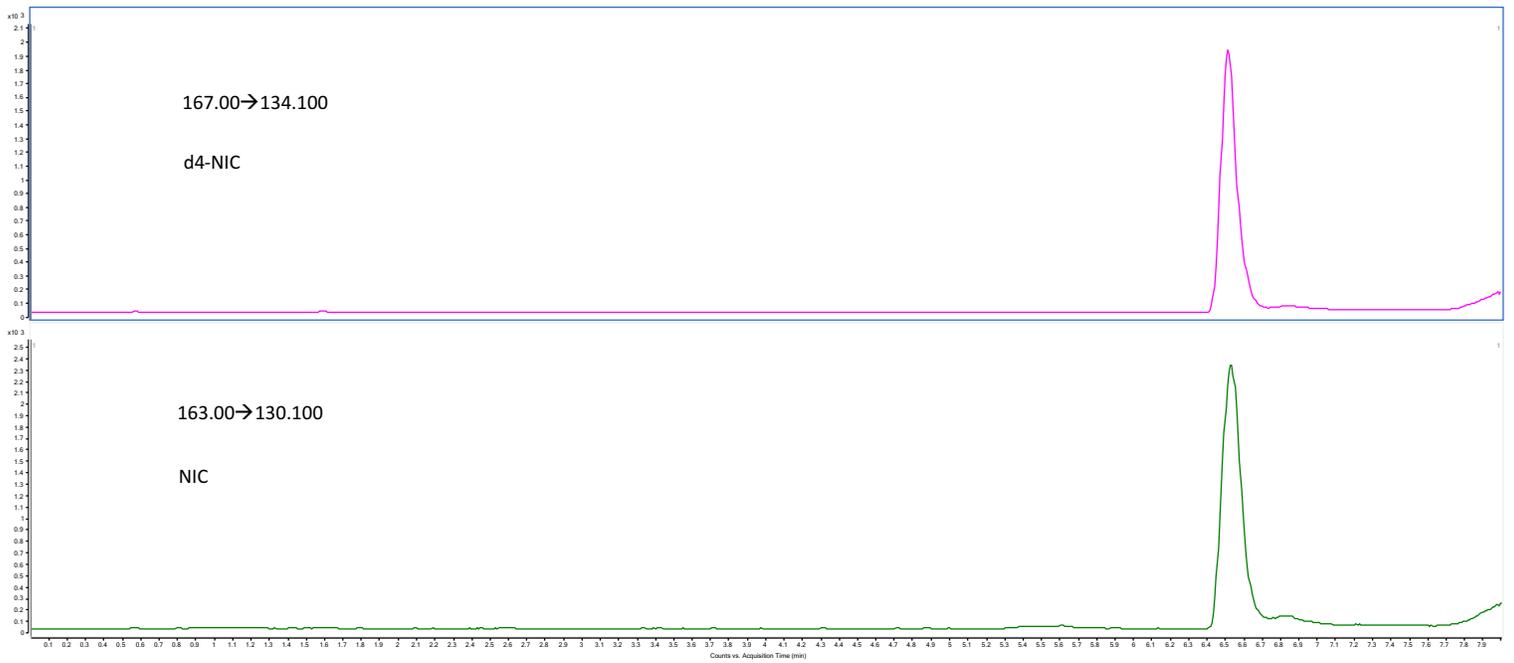


Figure S3. Method LC1 chromatogram of an extract of SRM 3222, with MRM transitions for nicotine and nicotine-d4

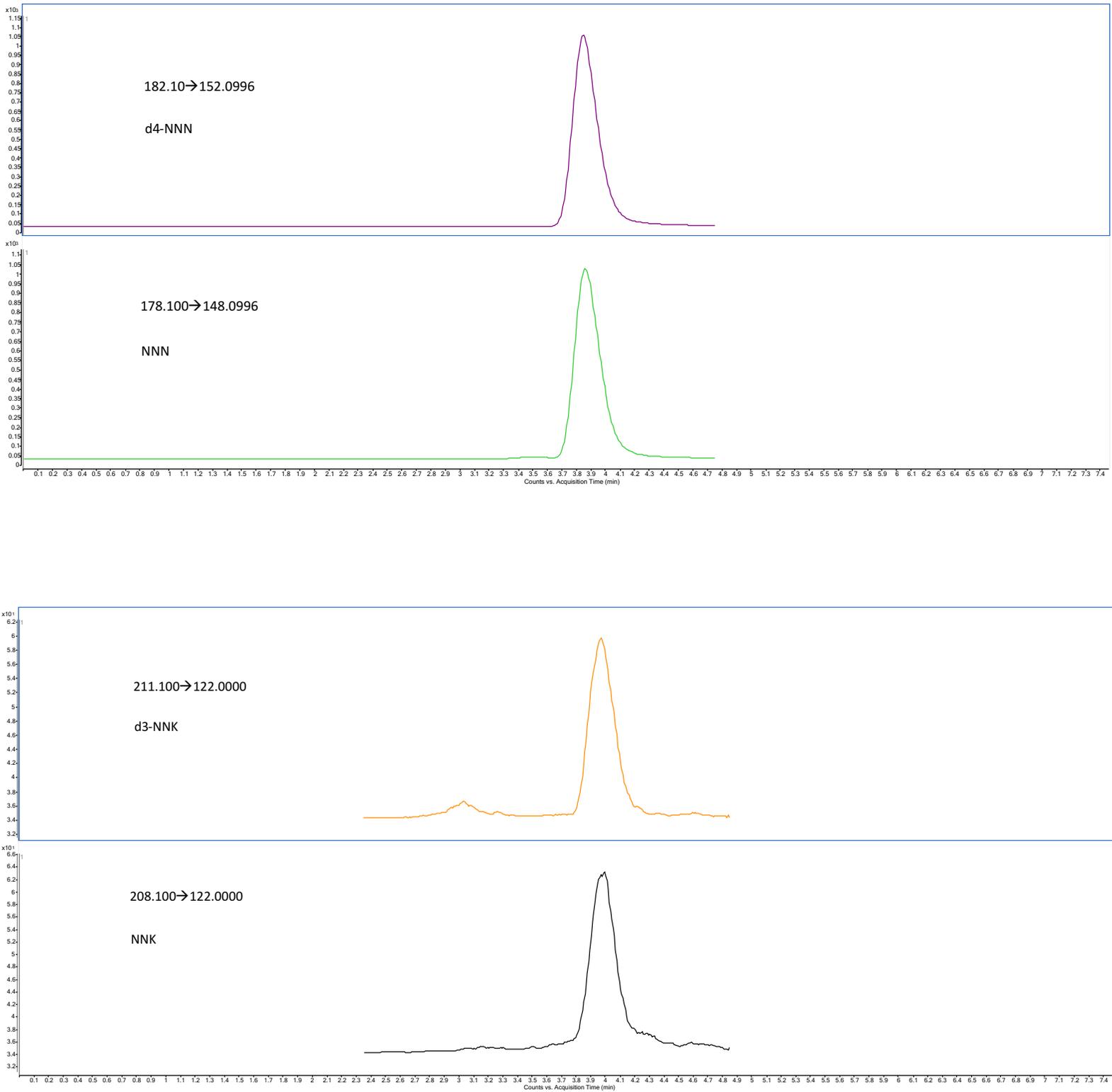


Figure S4. Method LC2 chromatograms of an extract of SRM 3222, with MRM transitions for NNN, d4-NNN, NNK, and d3-NNK

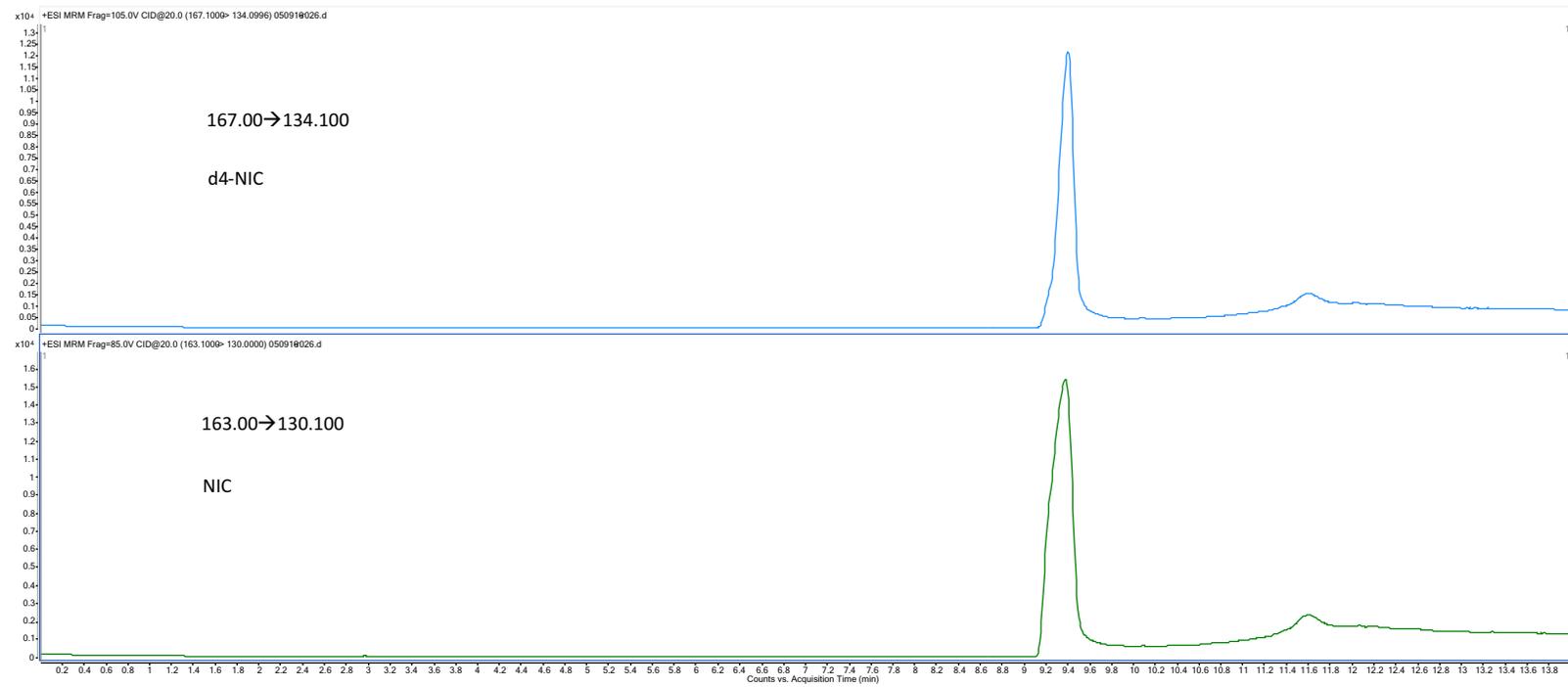


Figure S5. Method LC3 chromatogram of an extract of SRM 3222, with MRM transitions for nicotine and nicotine-d4

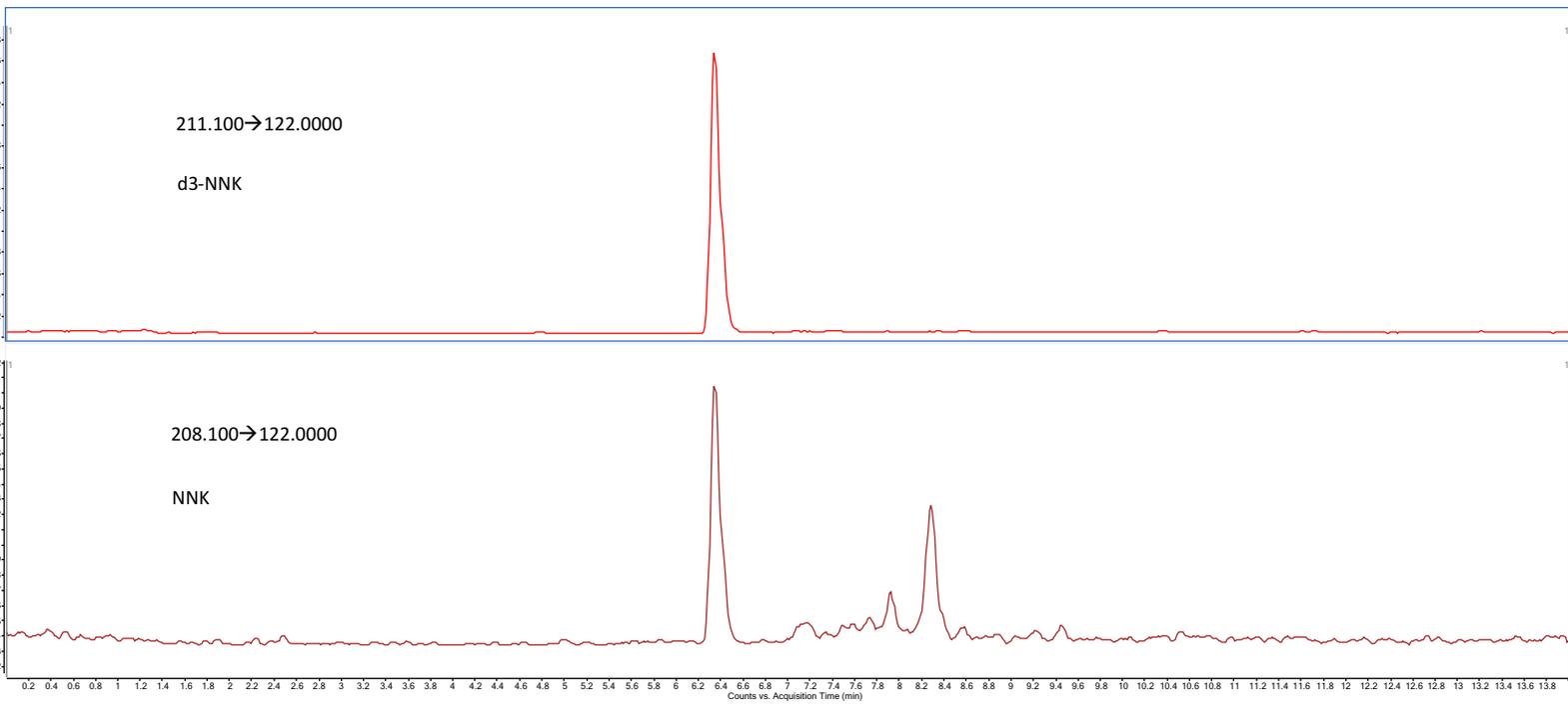
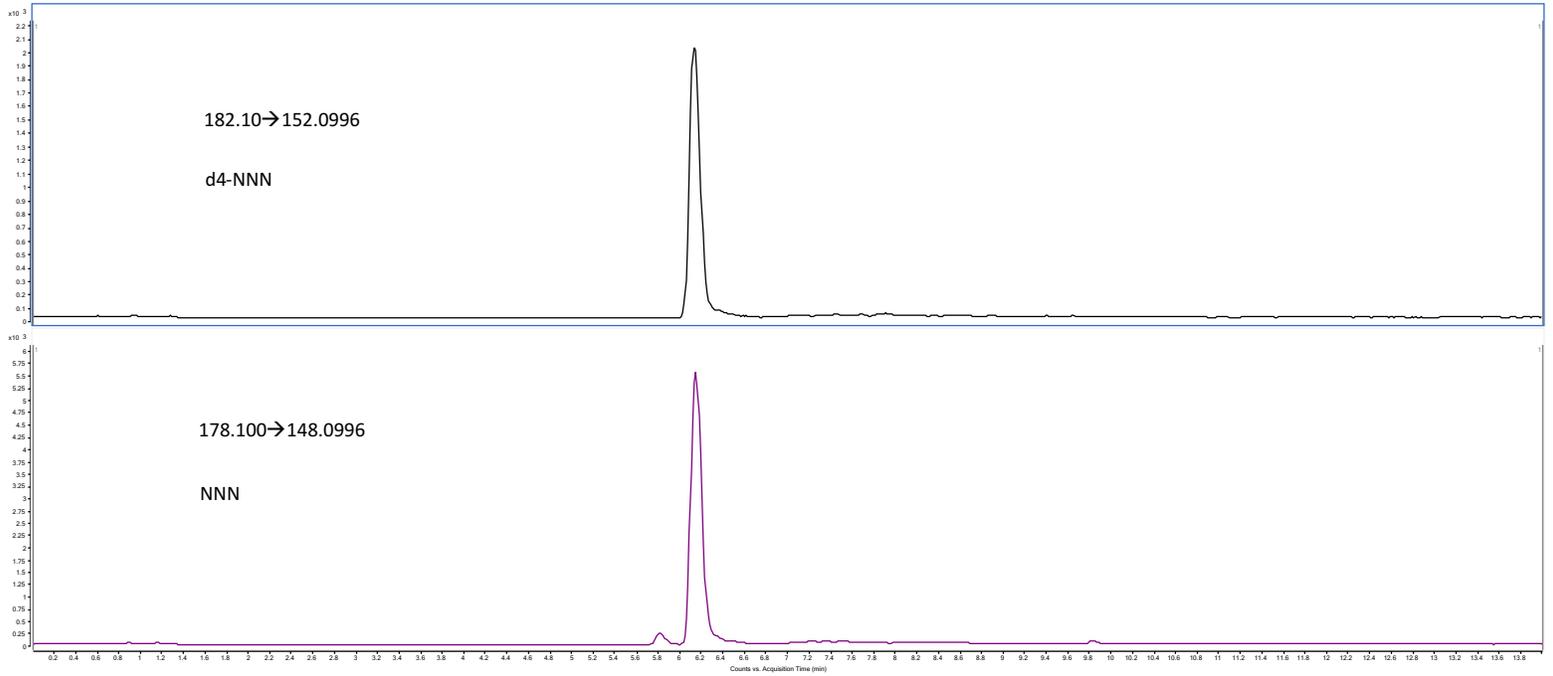


Figure S6. Method LC4 chromatograms of an extract of SRM 3222, with MRM transitions for NNN, d4-NNN, NNK, and d3-NNK

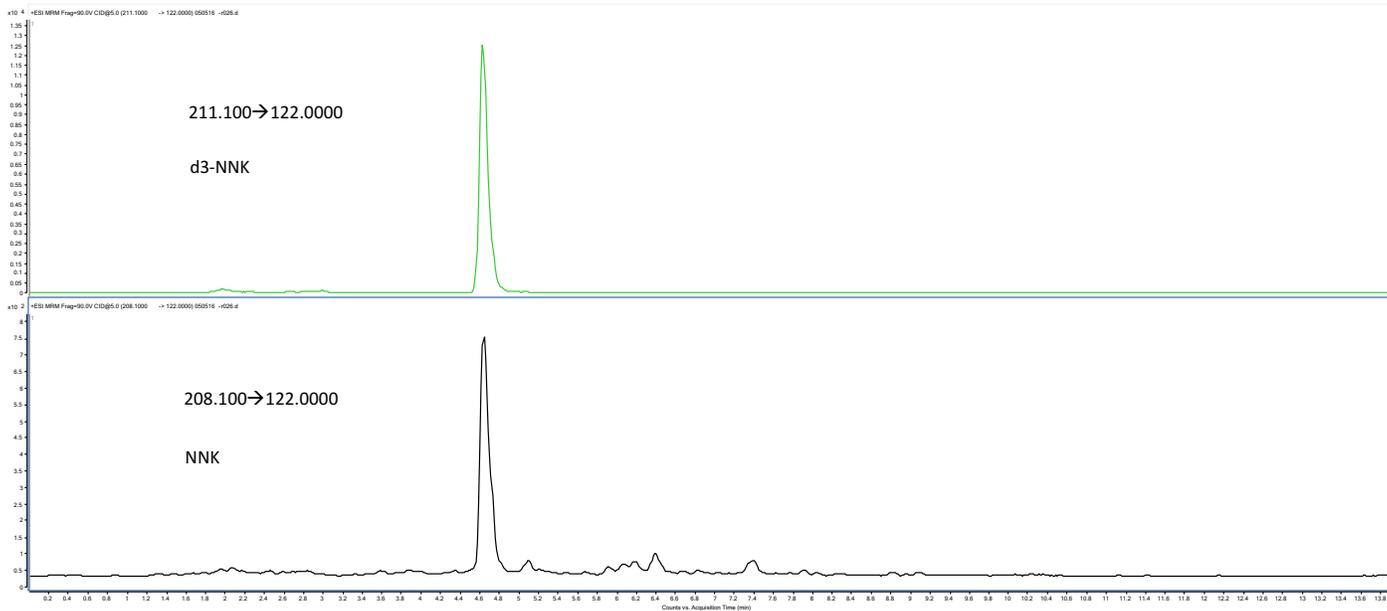
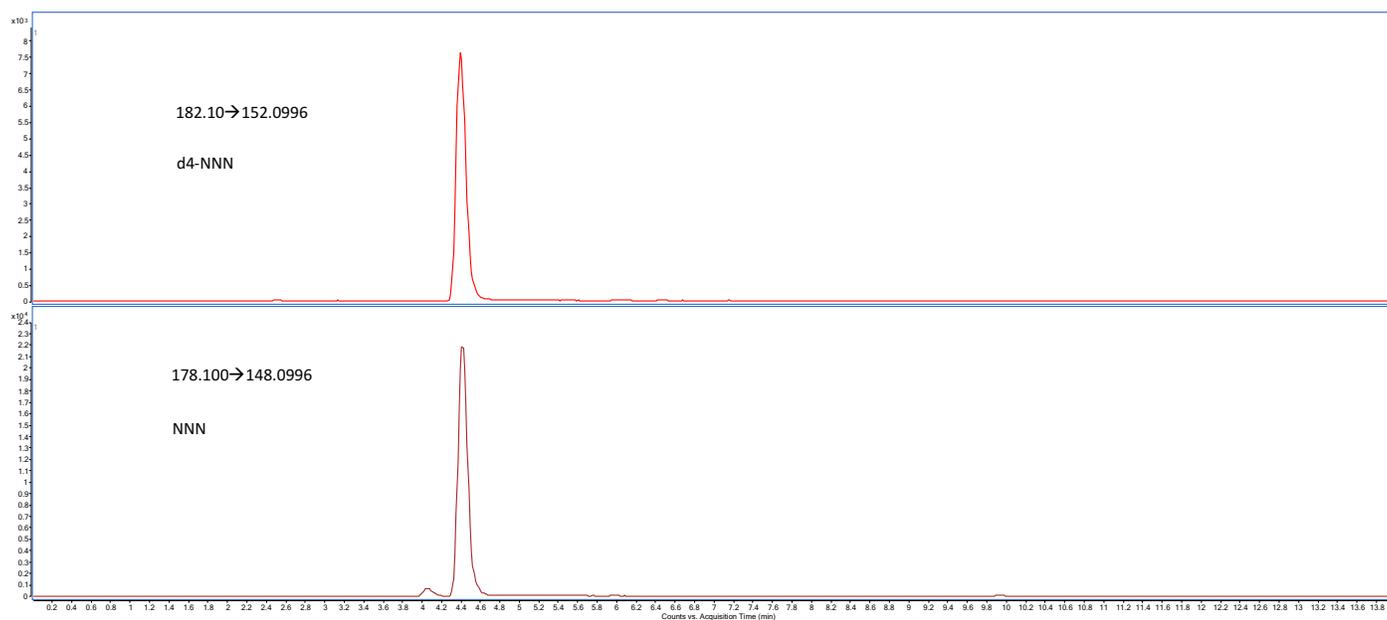


Figure S7. Method LC5 chromatograms of an extract of SRM 3222, with MRM transitions for NNN, d4-NNN, NNK, and d3-NNK

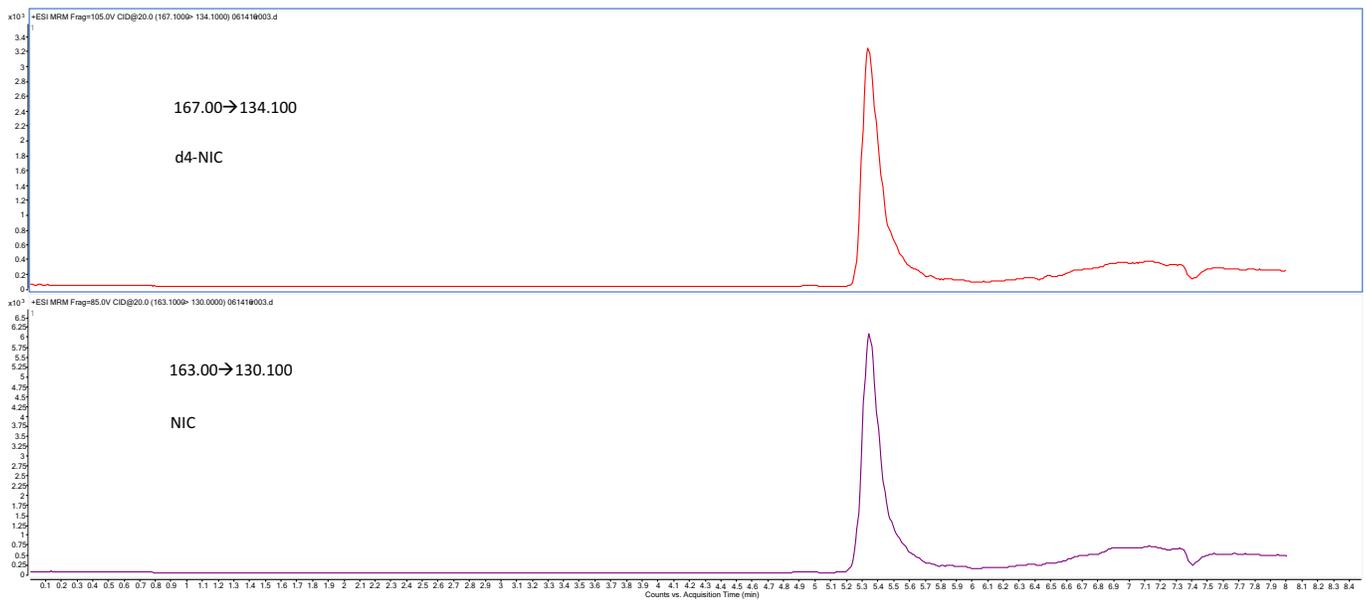


Figure S8. Method LC6 chromatogram of an extract of SRM 3222, with MRM transitions for nicotine and nicotine-d4

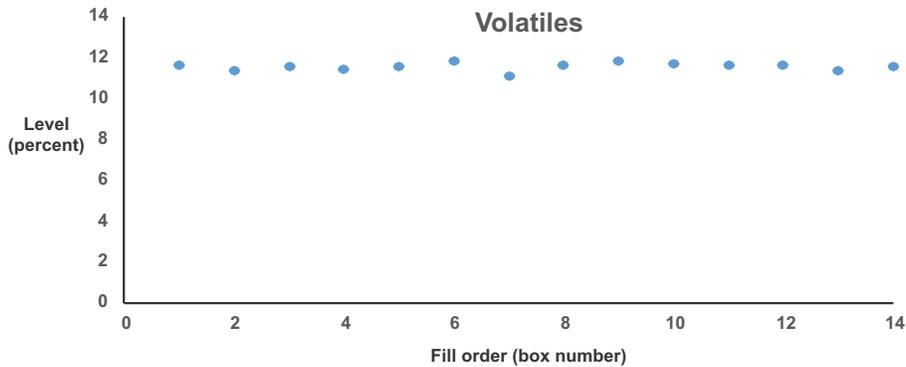
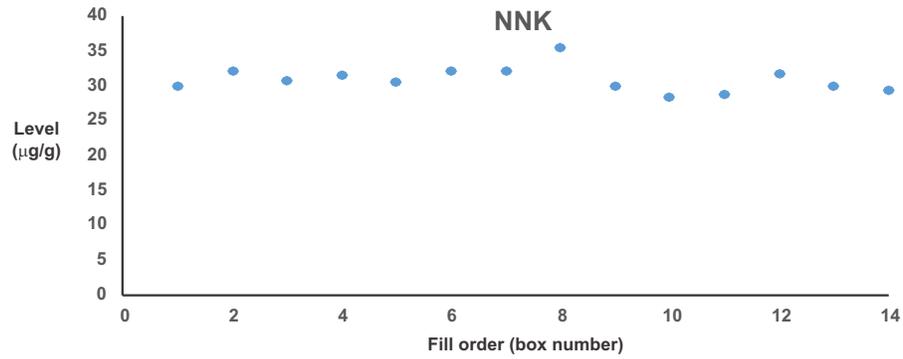
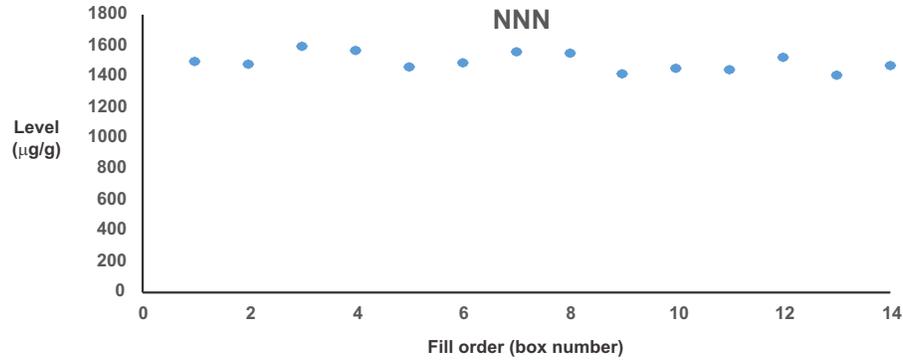
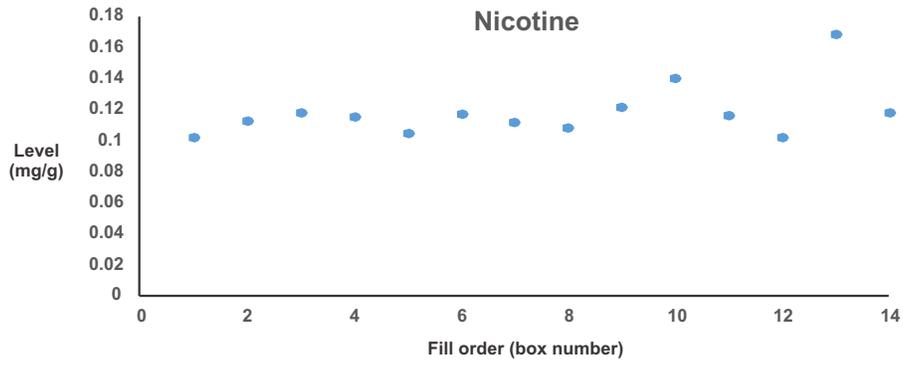


Figure S9. Levels of nicotine, nitrosamines, and volatiles plotted as a function of fill order, for samples selected by stratified random selection.

# Stability Study: Dry Mass Basis

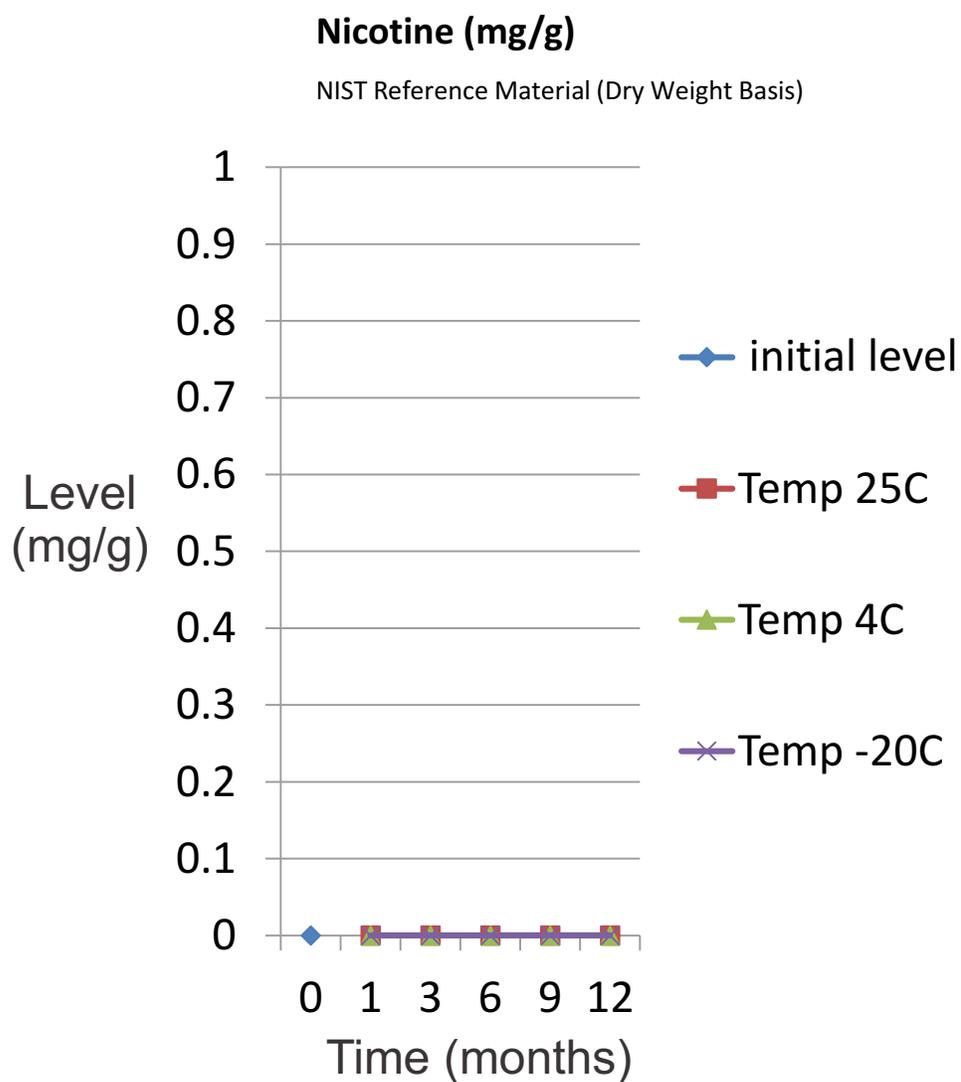


Figure S10. Nicotine levels (mg/g), plotted as a function of time (months), for SRM 3222 samples stored at different temperatures. Nicotine levels in SRM 3222 were below detectable limits for this study (see text).

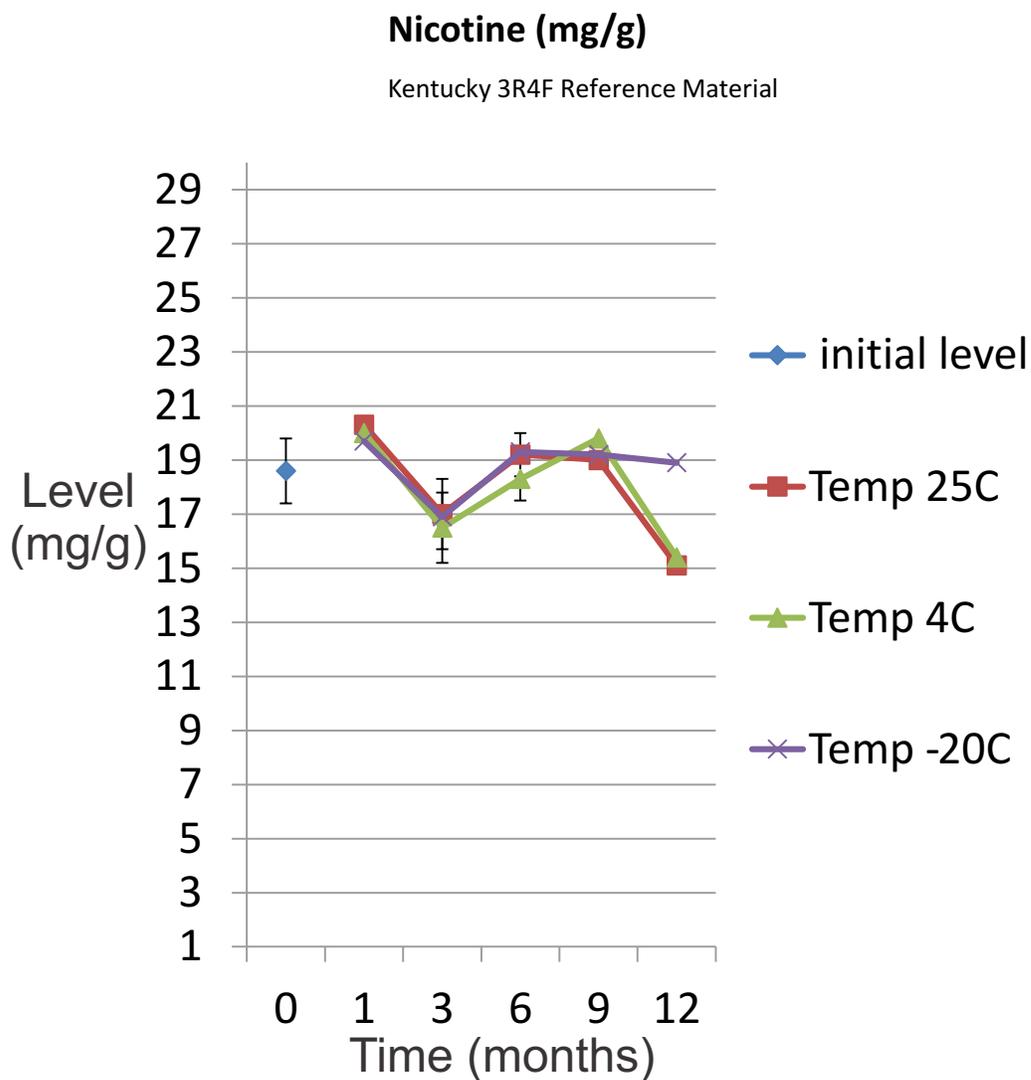


Figure S11. Nicotine levels (mg/g), plotted as a function of time (months), for Kentucky 3R4F Reference Material samples stored at different temperatures. Bars represent approximate 95% confidence intervals for 7 replicate measurements.

### Nitrosonornicotine (NNN) (ng/g)

NIST Reference Material

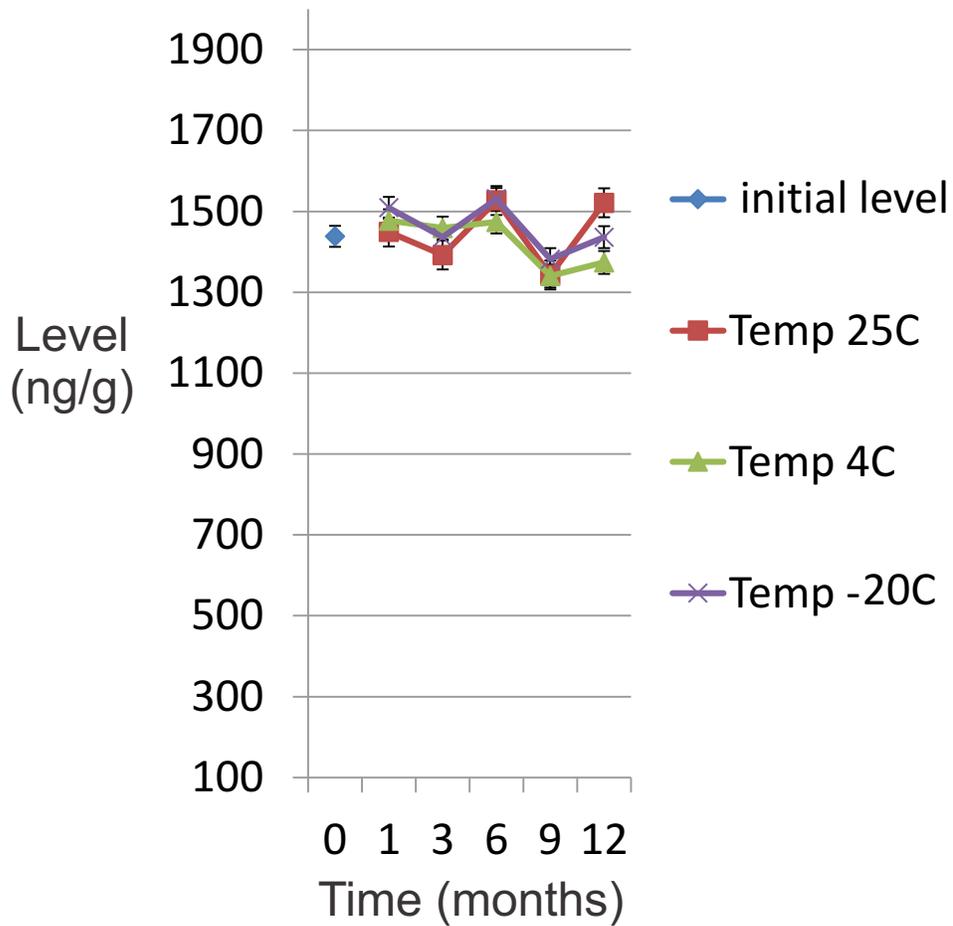


Figure S12. NNN levels (ng/g), plotted as a function of time (months), for SRM 3222 samples stored at different temperatures. Bars represent approximate 95% confidence intervals for 7 replicate measurements.

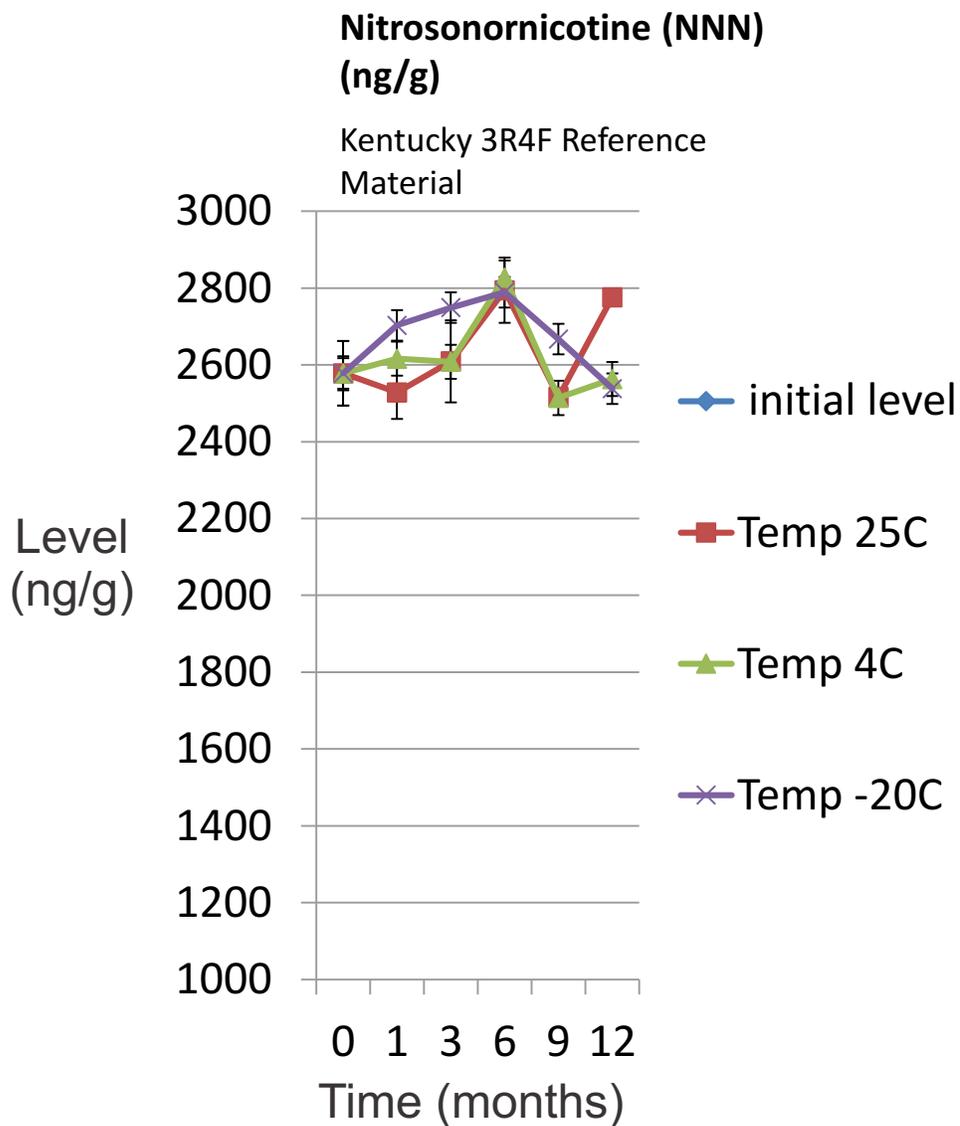


Figure S13. NNN levels (ng/g), plotted as a function of time (months), for Kentucky 3R4F Reference Material samples stored at different temperatures. Bars represent approximate 95% confidence intervals for 7 replicate measurements.

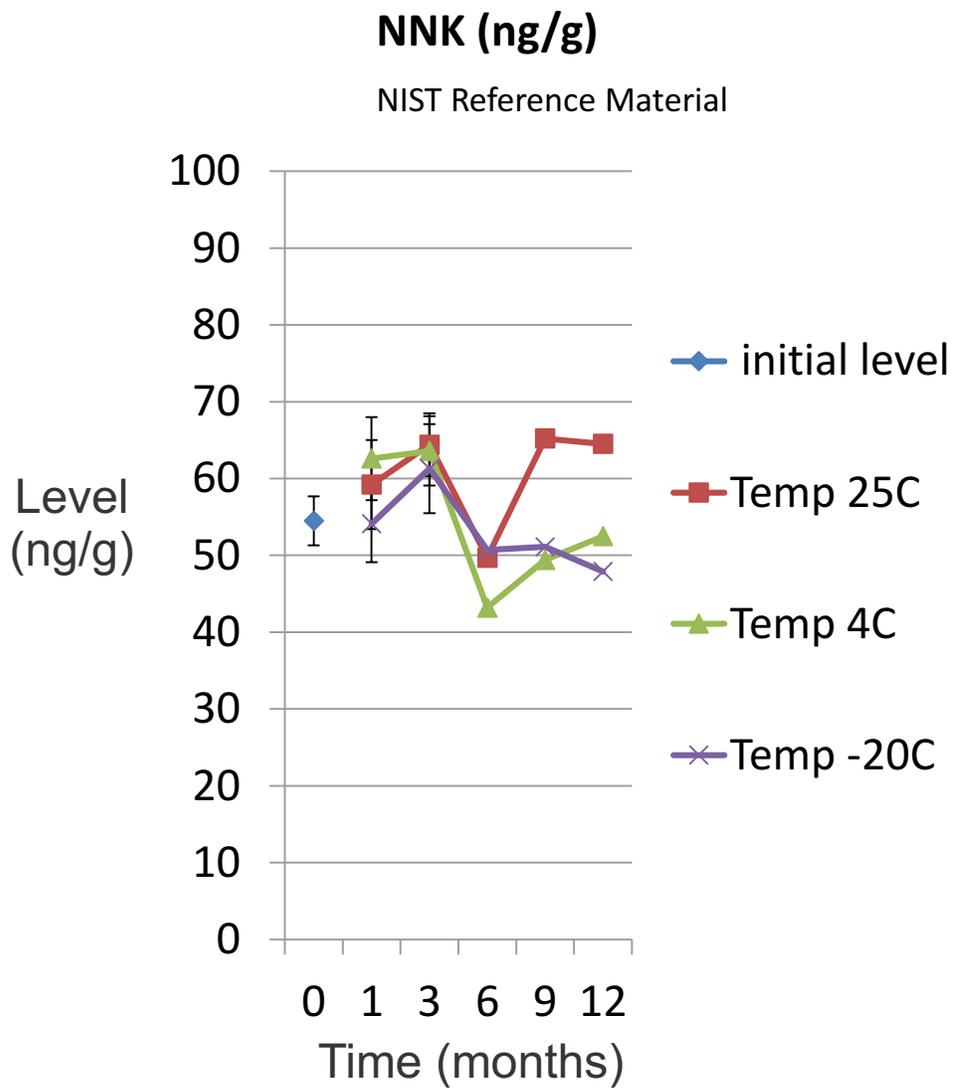


Figure S14. NNK levels (ng/g), plotted as a function of time (months), for SRM 3222 samples stored at different temperatures. Bars represent approximate 95% confidence intervals for 7 replicate measurements.

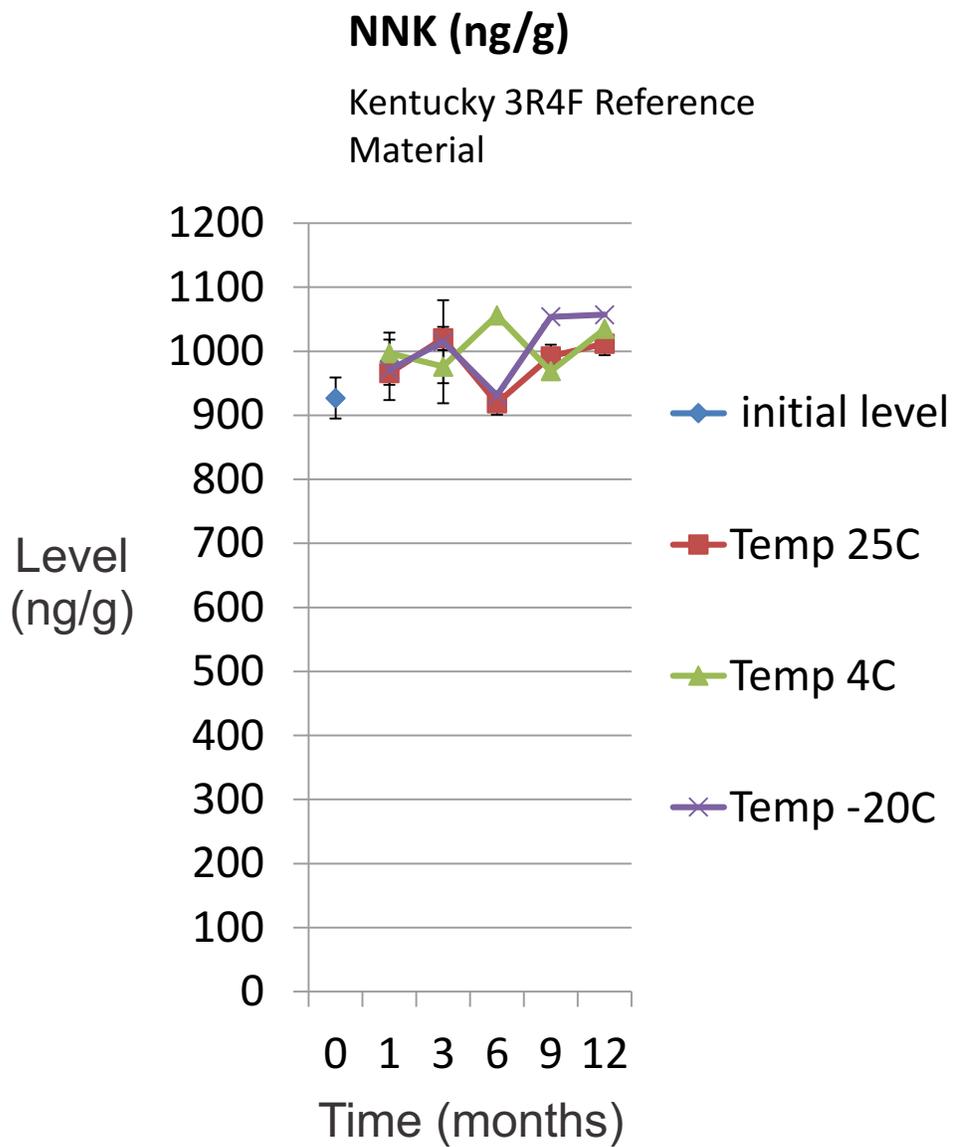


Figure S15. NNK levels (ng/g), plotted as a function of time (months), for Kentucky 3R4F Reference Material samples stored at different temperatures. Bars represent approximate 95% confidence intervals for 7 replicate measurements.

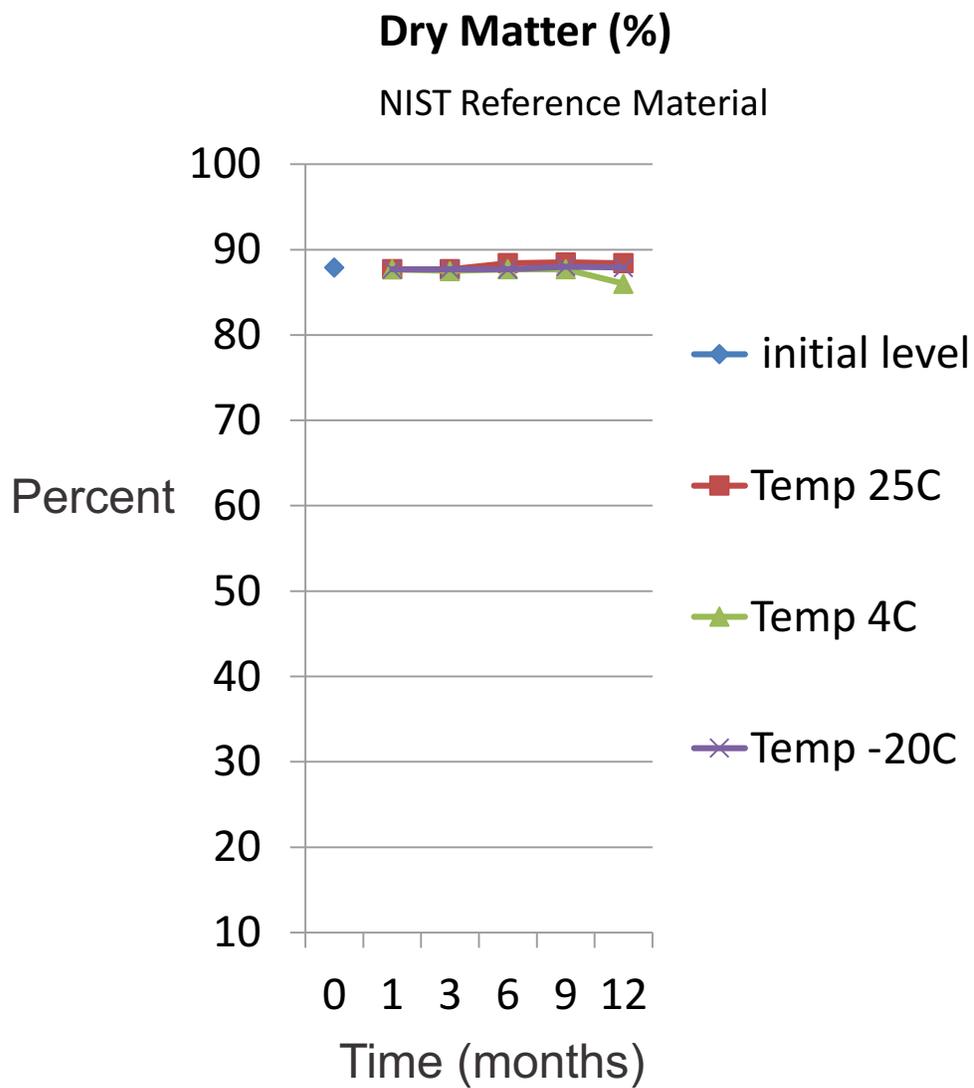


Figure S16. Dry matter (percent), plotted as a function of time (months), for SRM 3222 samples stored at different temperatures.

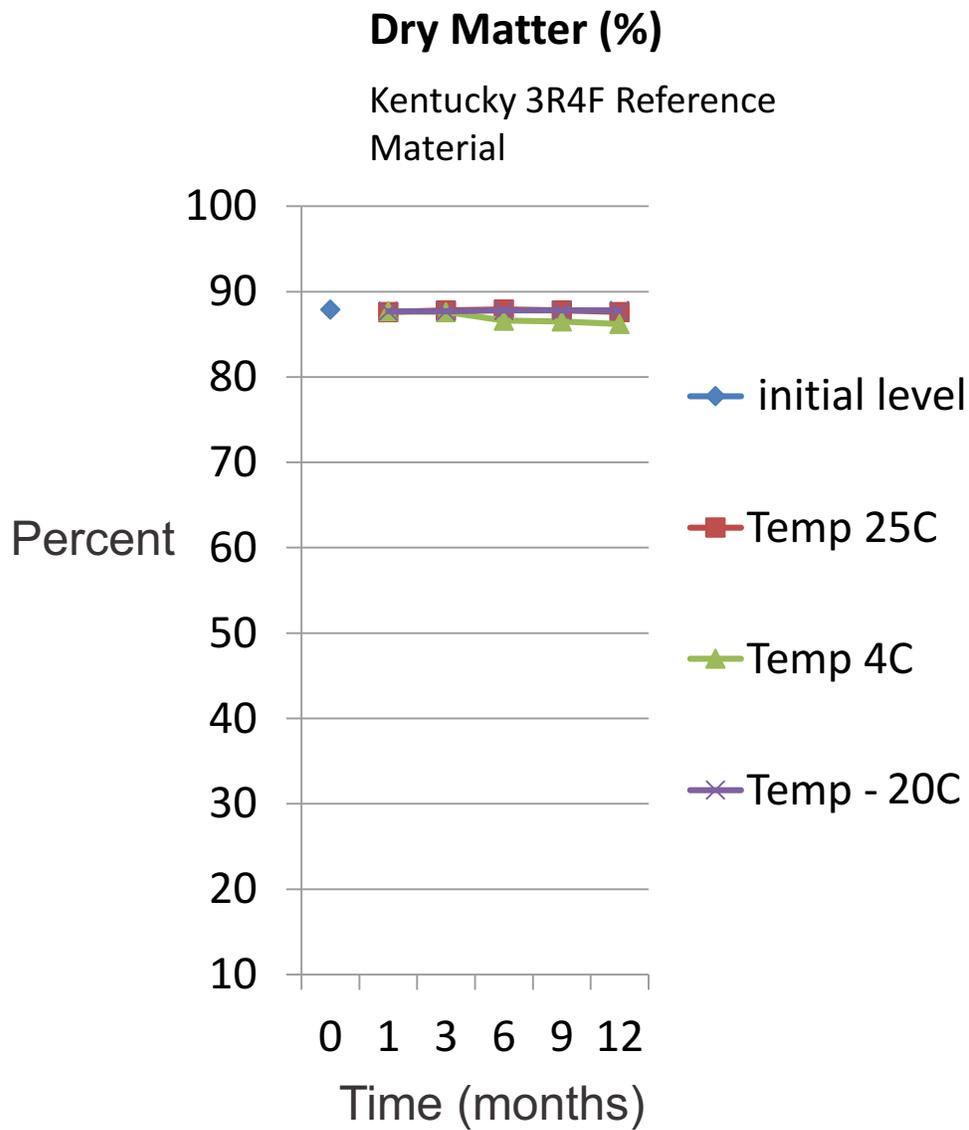


Figure S17. Dry matter (percent), plotted as a function of time (months), for Kentucky 3R4F Reference Material samples stored at different temperatures.

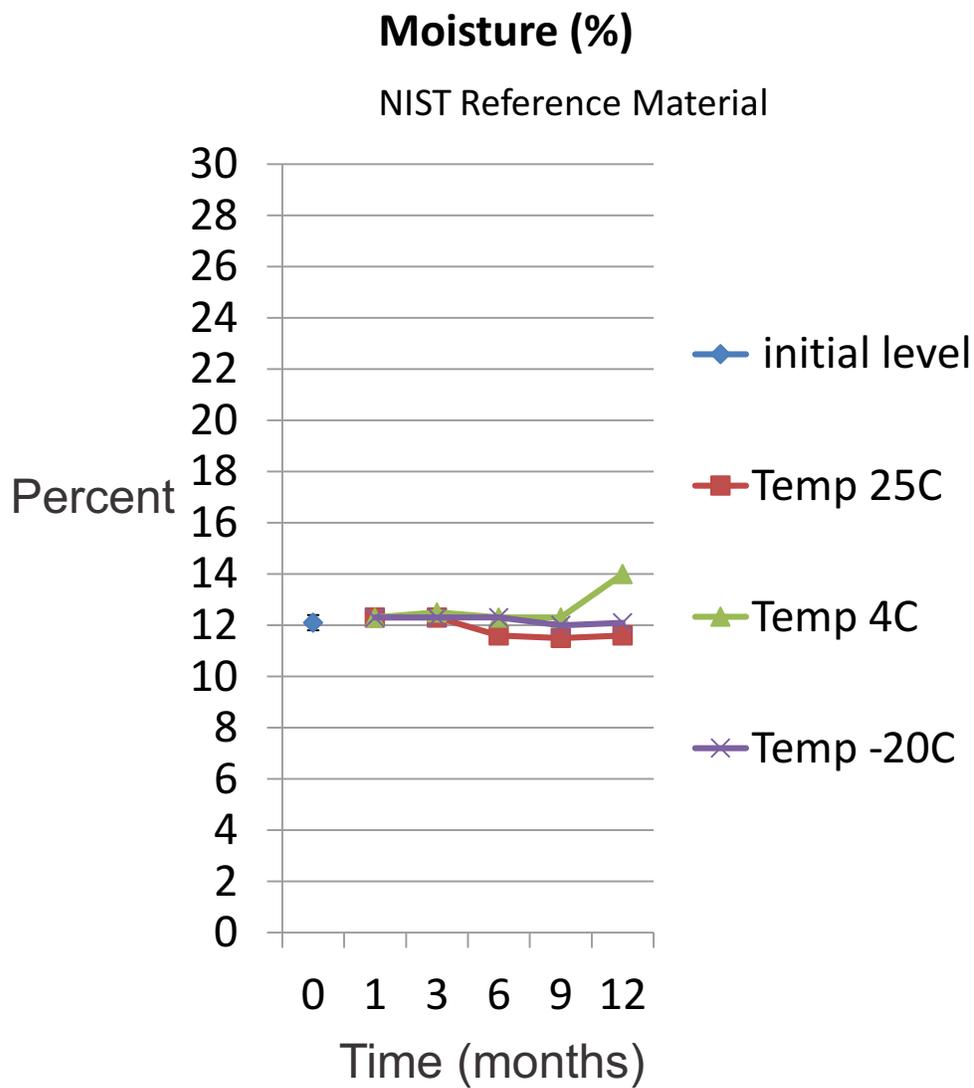


Figure S18. Moisture (percent), plotted as a function of time (months), for SRM 3222 samples stored at different temperatures.

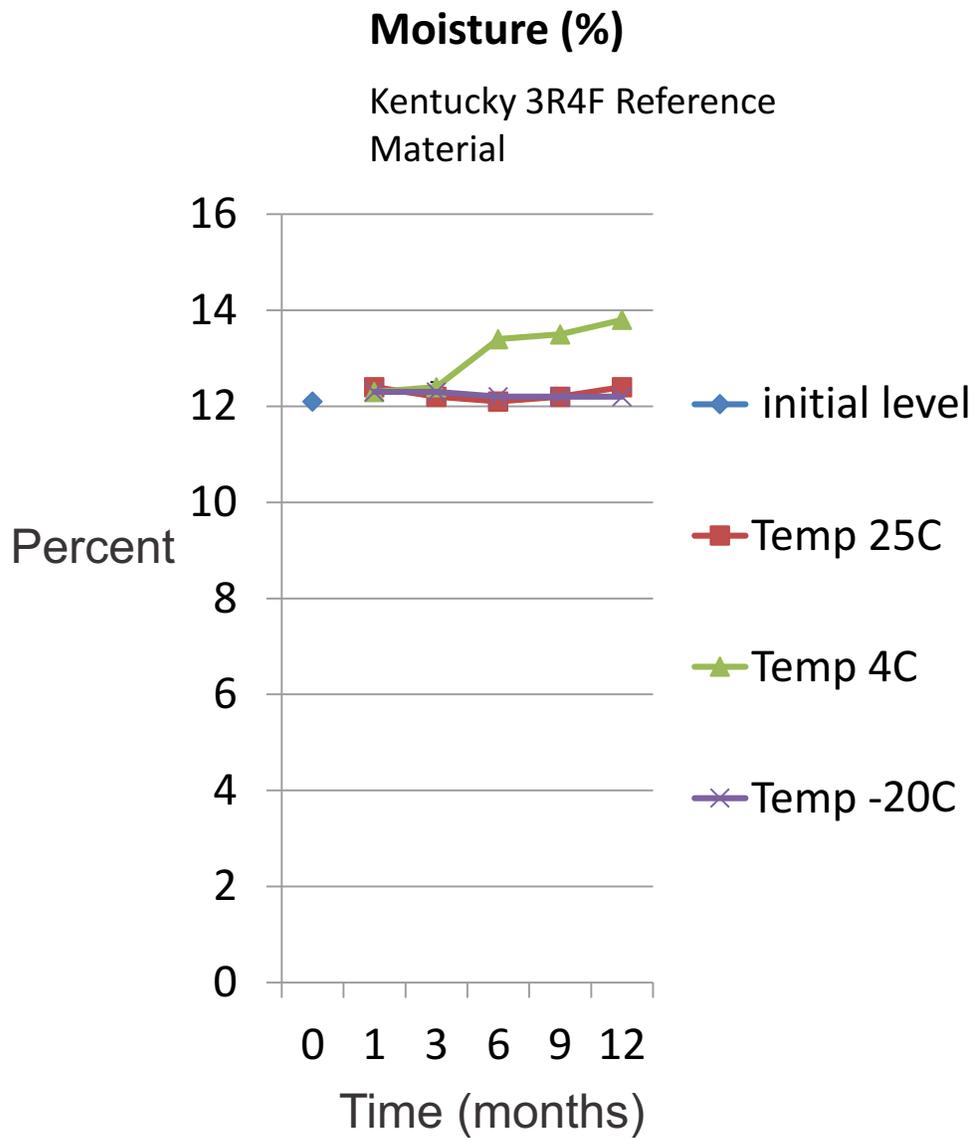


Figure S19. Moisture (percent), plotted as a function of time (months), for Kentucky 3R4F Reference Material samples stored at different temperatures.

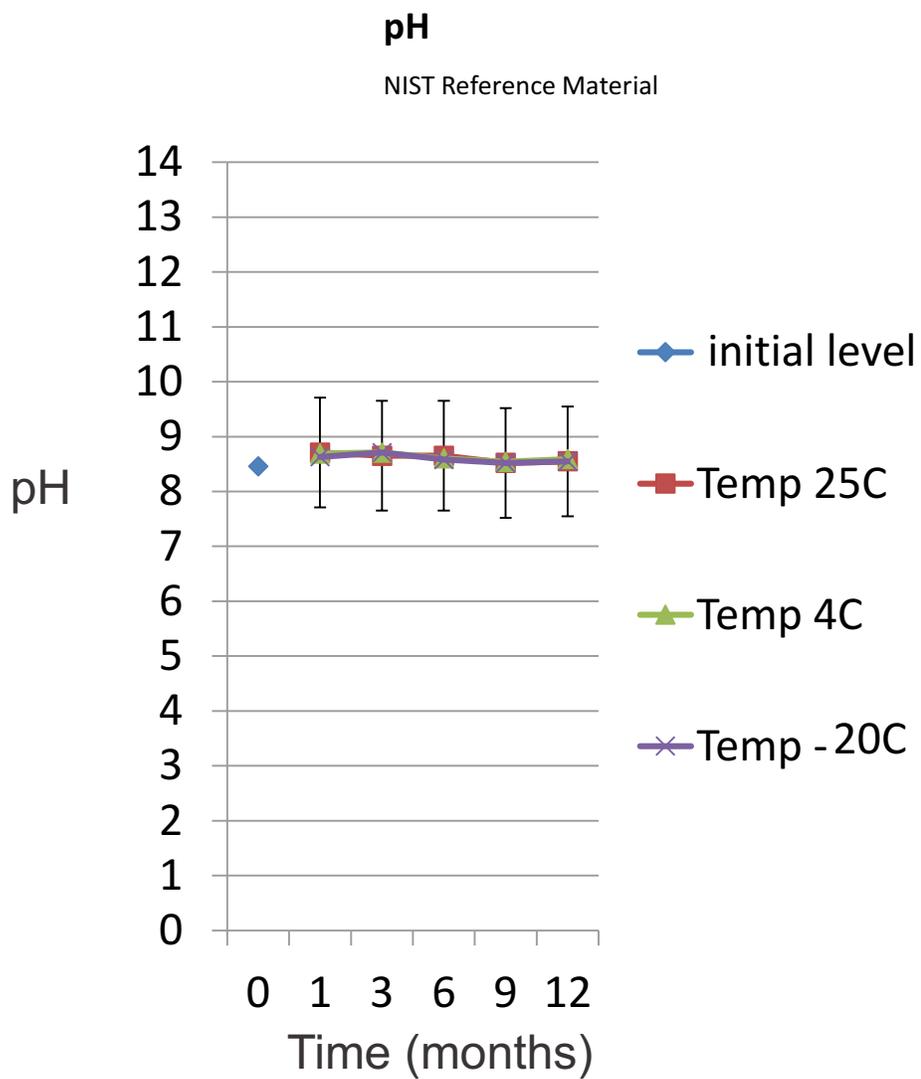


Figure S20. pH, plotted as a function of time (months), for SRM 3222 samples stored at different temperatures. Bars represent approximate 95% confidence intervals for 7 replicate measurements.

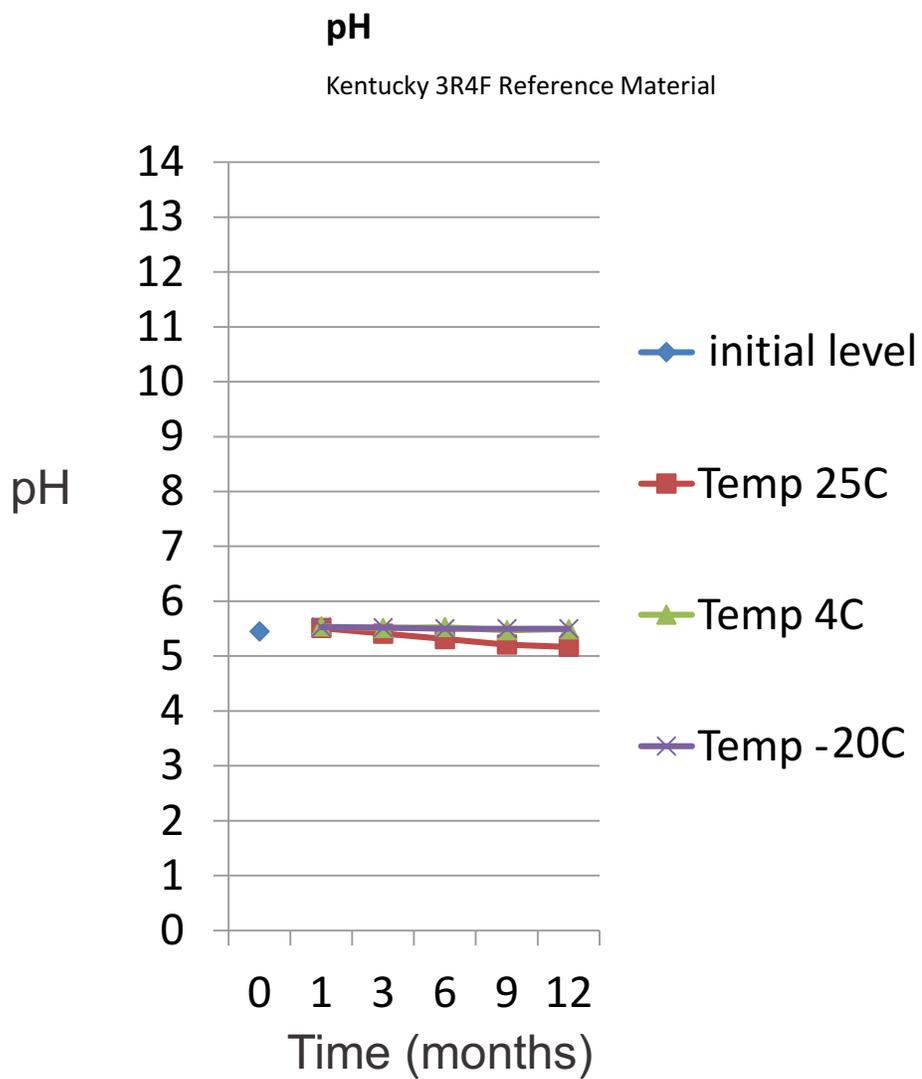


Figure S21. pH, plotted as a function of time (months), for Kentucky 3R4F Reference Material samples stored at different temperatures.

### Freeze/Thaw Cycle: Nicotine (mg/g)

Kentucky 3R4F Reference Material  
NIST Reference Material

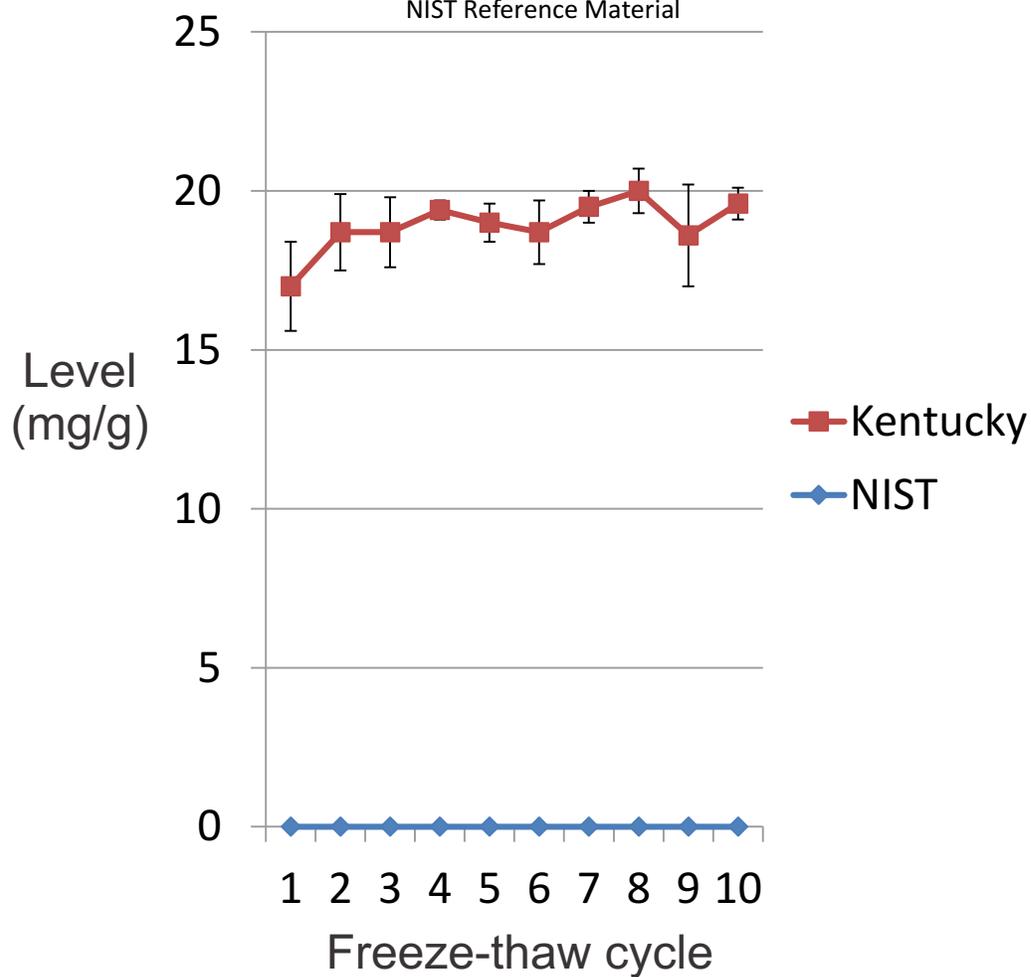


Figure S22. Nicotine levels (mg/g), plotted as a function of freeze-thaw cycle, for SRM 3222 and Kentucky 3R4F Reference Material samples. Nicotine levels in SRM 3222 were below detectable limits for this study (see text). Bars represent approximate 95% confidence intervals for 7 replicate

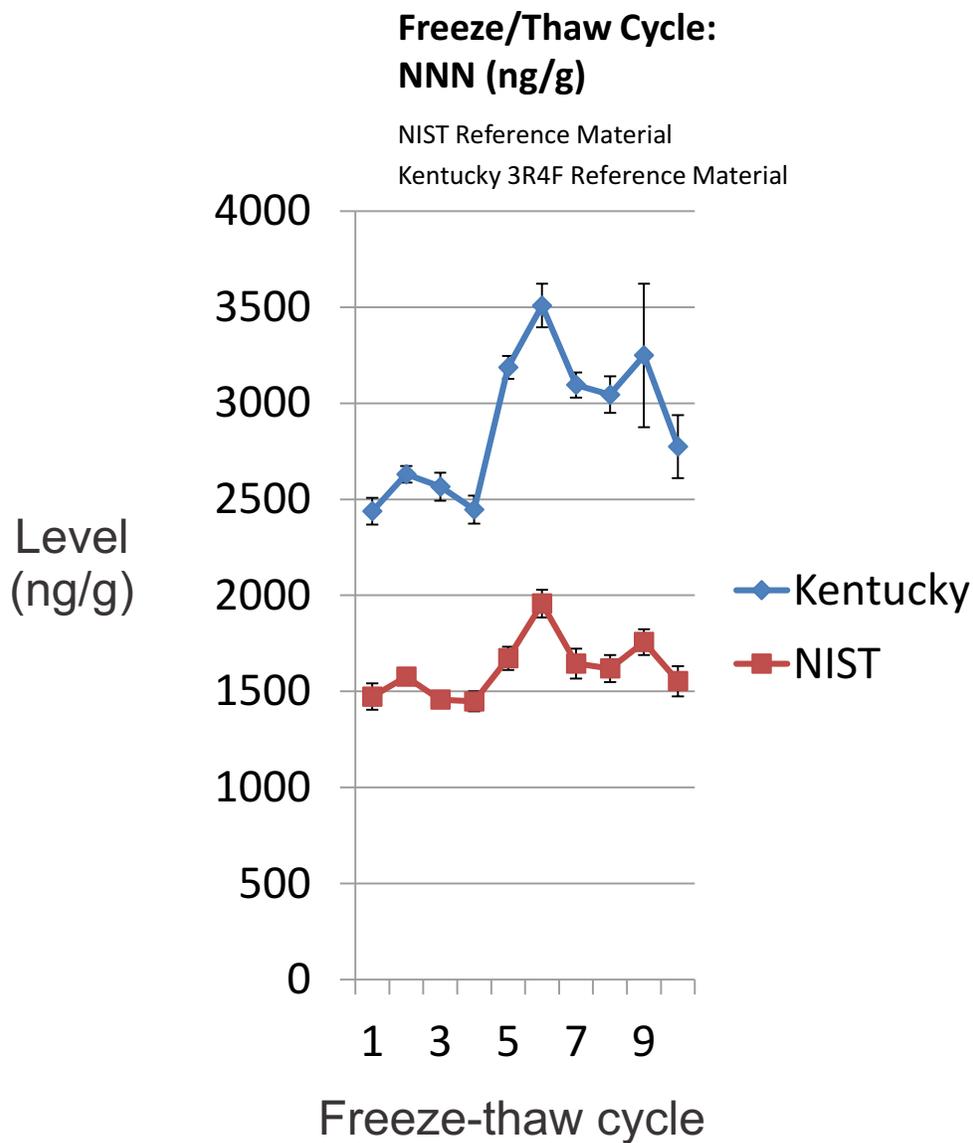


Figure S23. NNN levels (ng/g), plotted as a function of freeze-thaw cycle, for SRM 3222 and Kentucky 3R4F Reference Material samples. Bars represent approximate 95% confidence intervals for 7 replicate measurements.

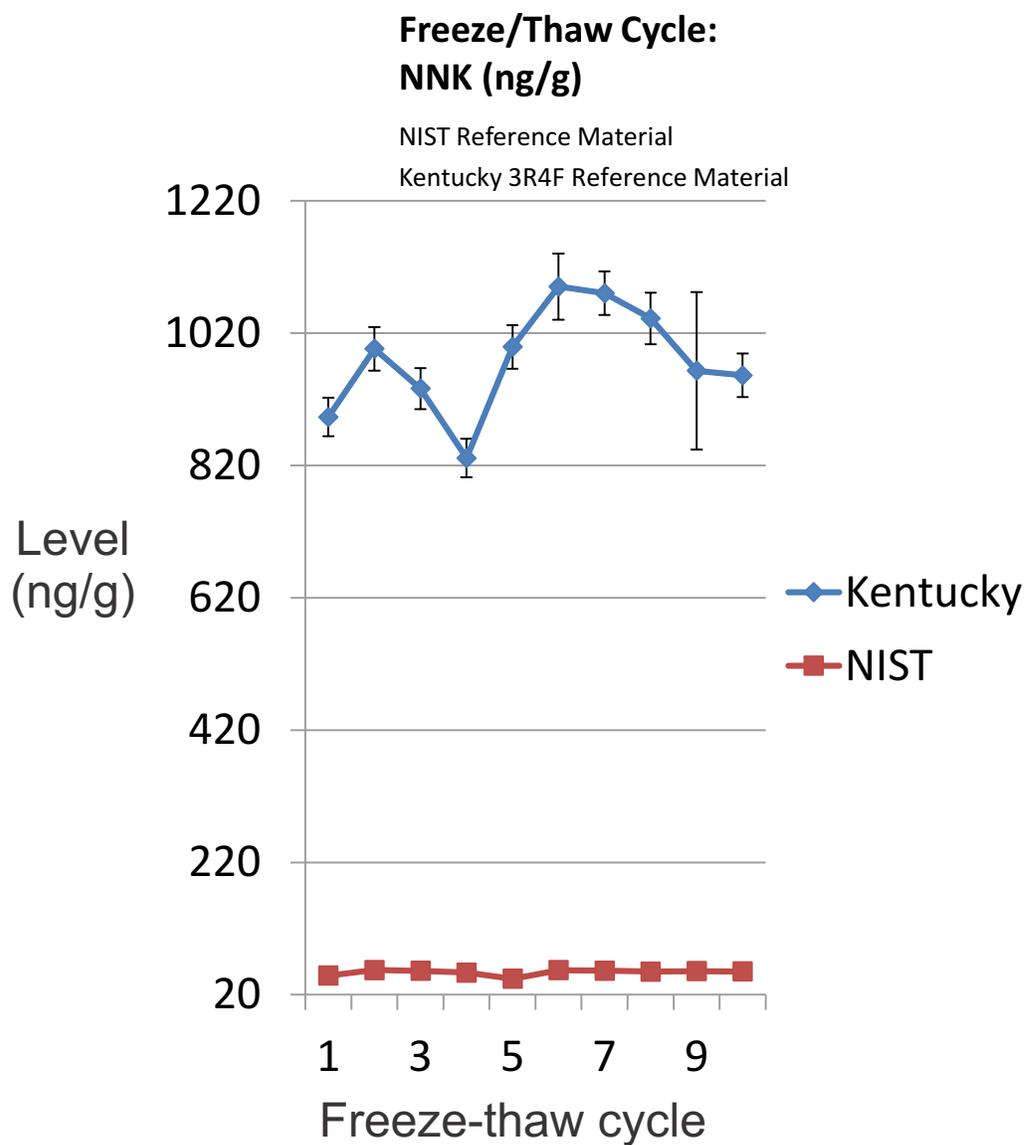


Figure S24. NNK levels (ng/g), plotted as a function of freeze-thaw cycle, for SRM 3222 and Kentucky 3R4F Reference Material samples. Bars represent approximate 95% confidence intervals for 7 replicate measurements.

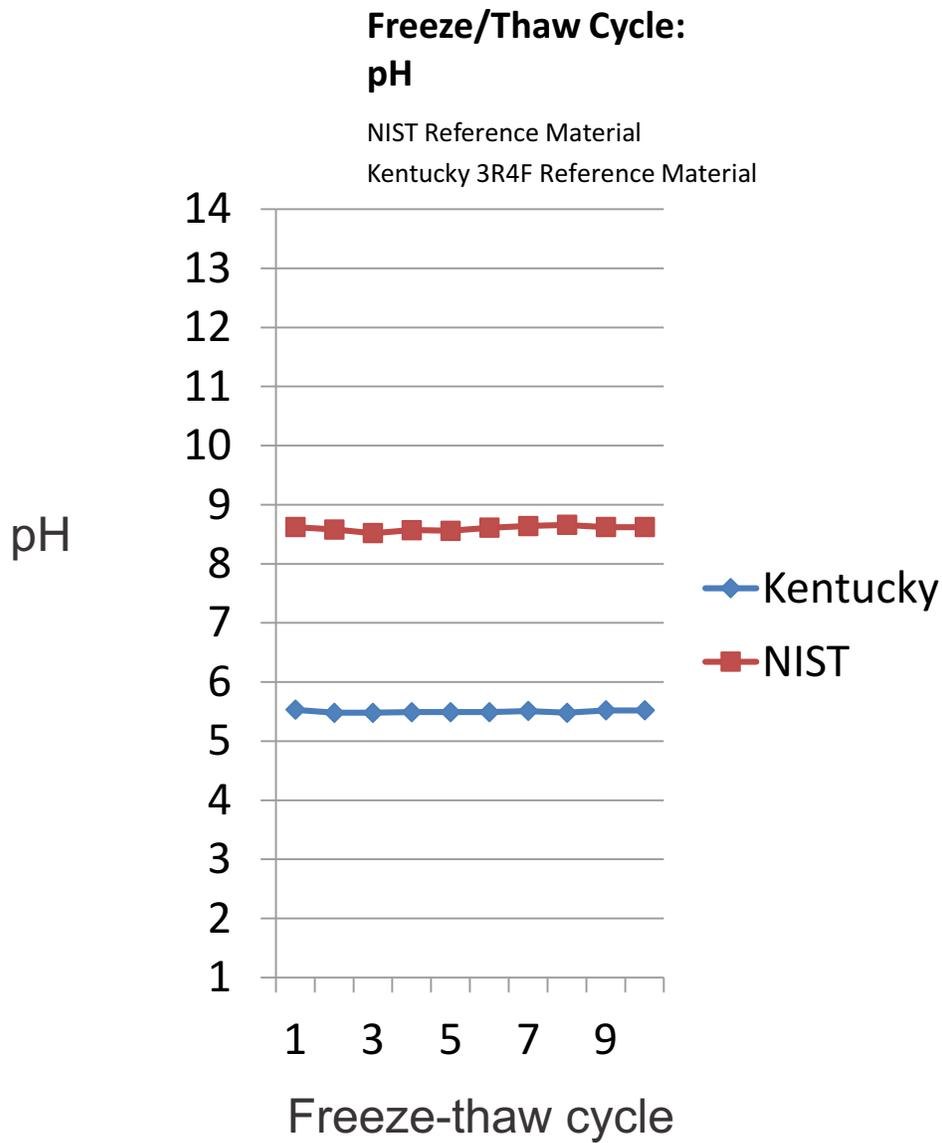


Figure S25. pH, plotted as a function of freeze-thaw cycle, for SRM 3222 and Kentucky 3R4F Reference Material samples.

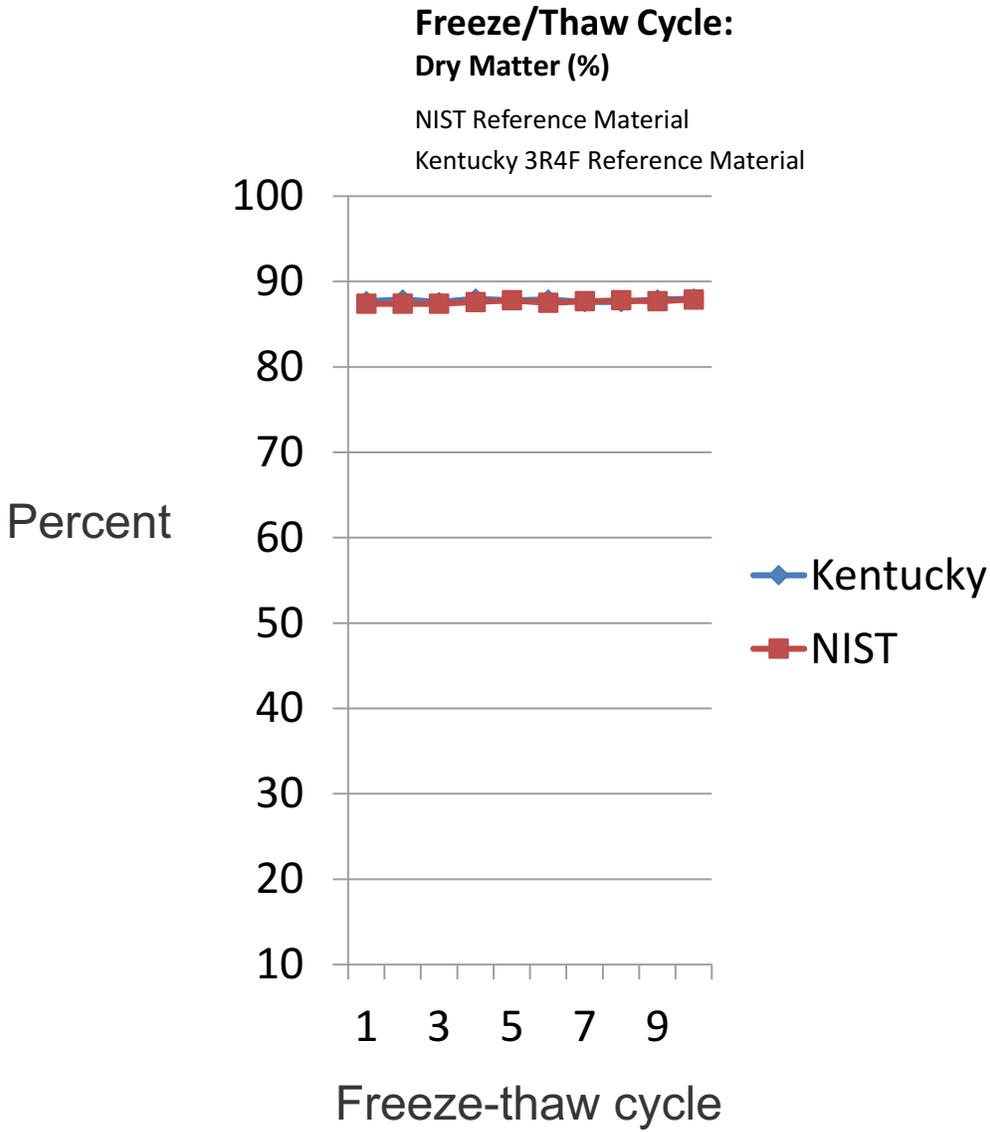


Figure S26. Dry Matter (percent), plotted as a function of freeze-thaw cycle, for SRM 3222 and Kentucky 3R4F Reference Material samples.

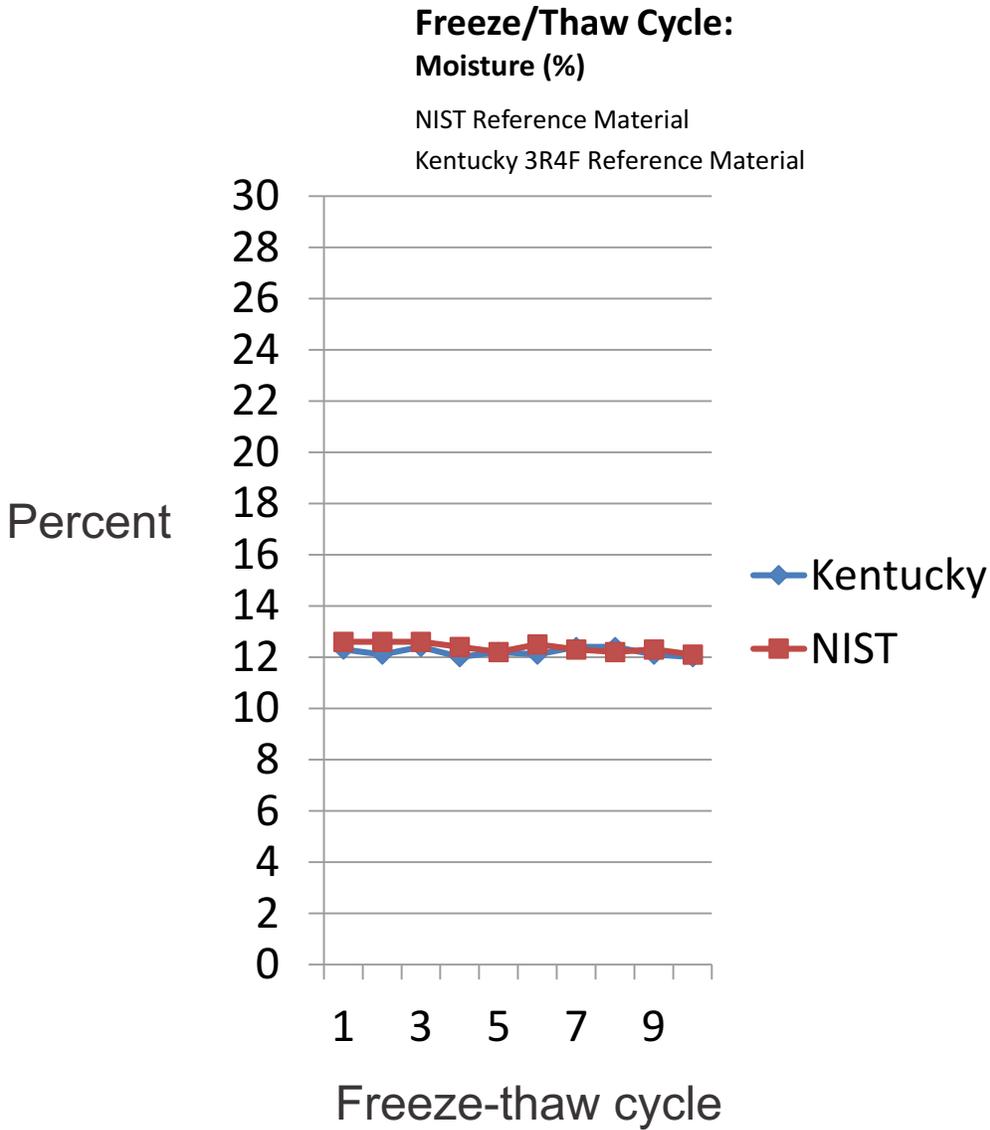


Figure S27. Moisture (percent), plotted as a function of freeze-thaw cycle, for SRM 3222 and Kentucky 3R4F Reference Material samples.

# **Stability Study: As-Received Basis**

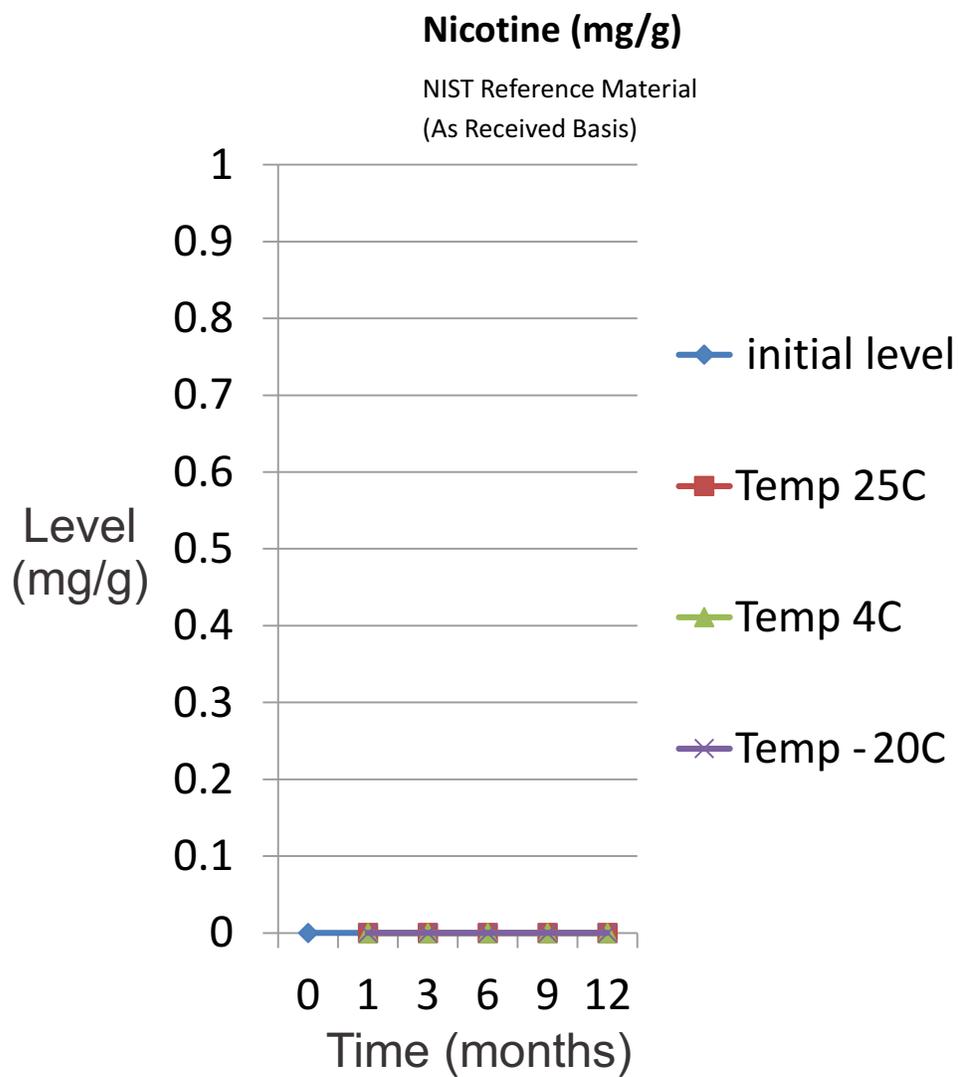


Figure S28. Nicotine levels (mg/g), plotted as a function of time (months), for SRM 3222 samples stored at different temperatures. Nicotine levels in SRM 3222 were below detectable limits for this study (see text).

### Nicotine (mg/g)

Kentucky 3R4F Reference Material

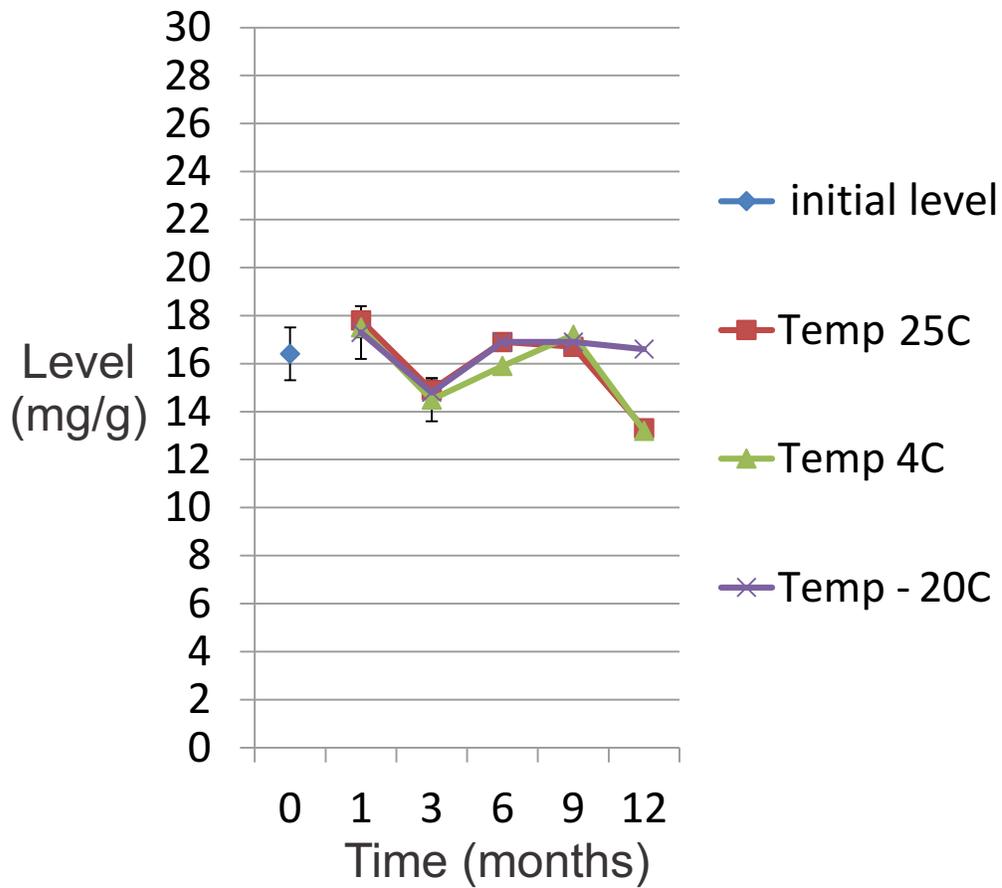


Figure S29. Nicotine levels (mg/g), plotted as a function of time (months), for Kentucky 3R4F Reference Material samples stored at different temperatures. Bars represent approximate 95% confidence intervals for 7 replicate measurements.

### Nitrosonornicotine (NNN) (ng/g)

NIST Reference Material

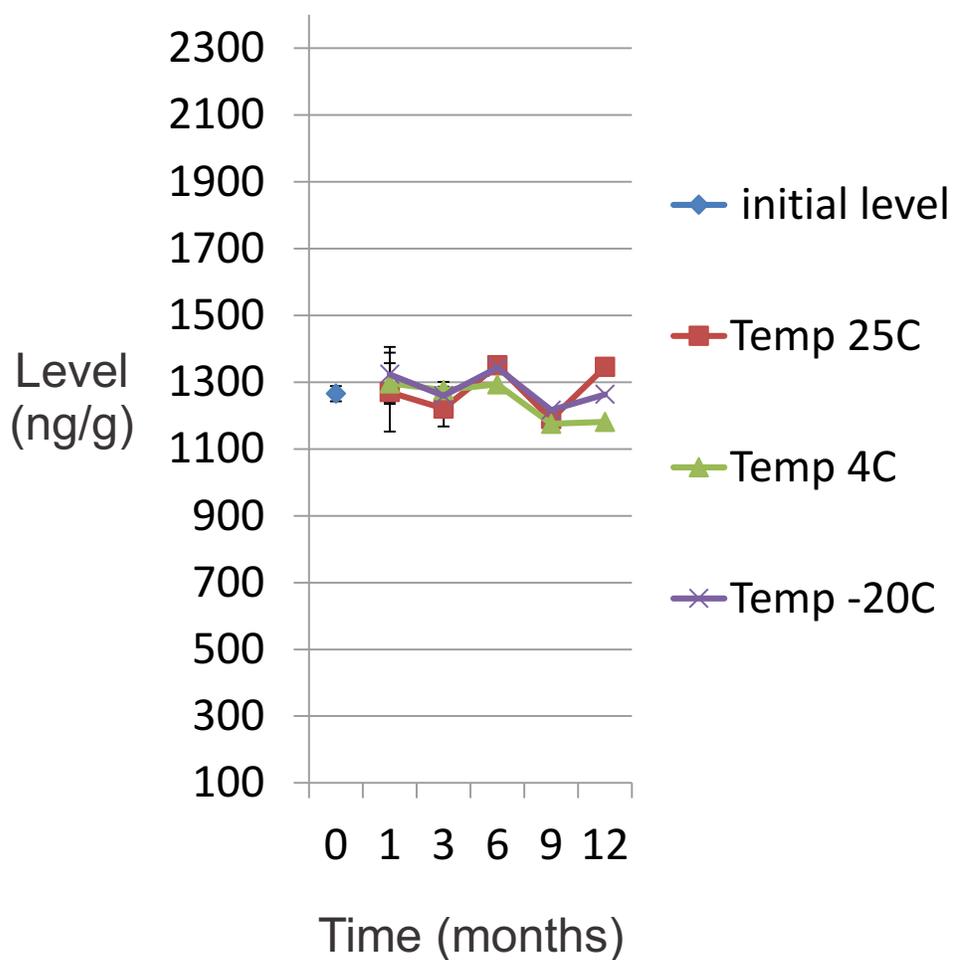


Figure S30. NNN levels (ng/g), plotted as a function of time (months), for SRM 3222 samples stored at different temperatures. Bars represent approximate 95% confidence intervals for 7 replicate measurements.

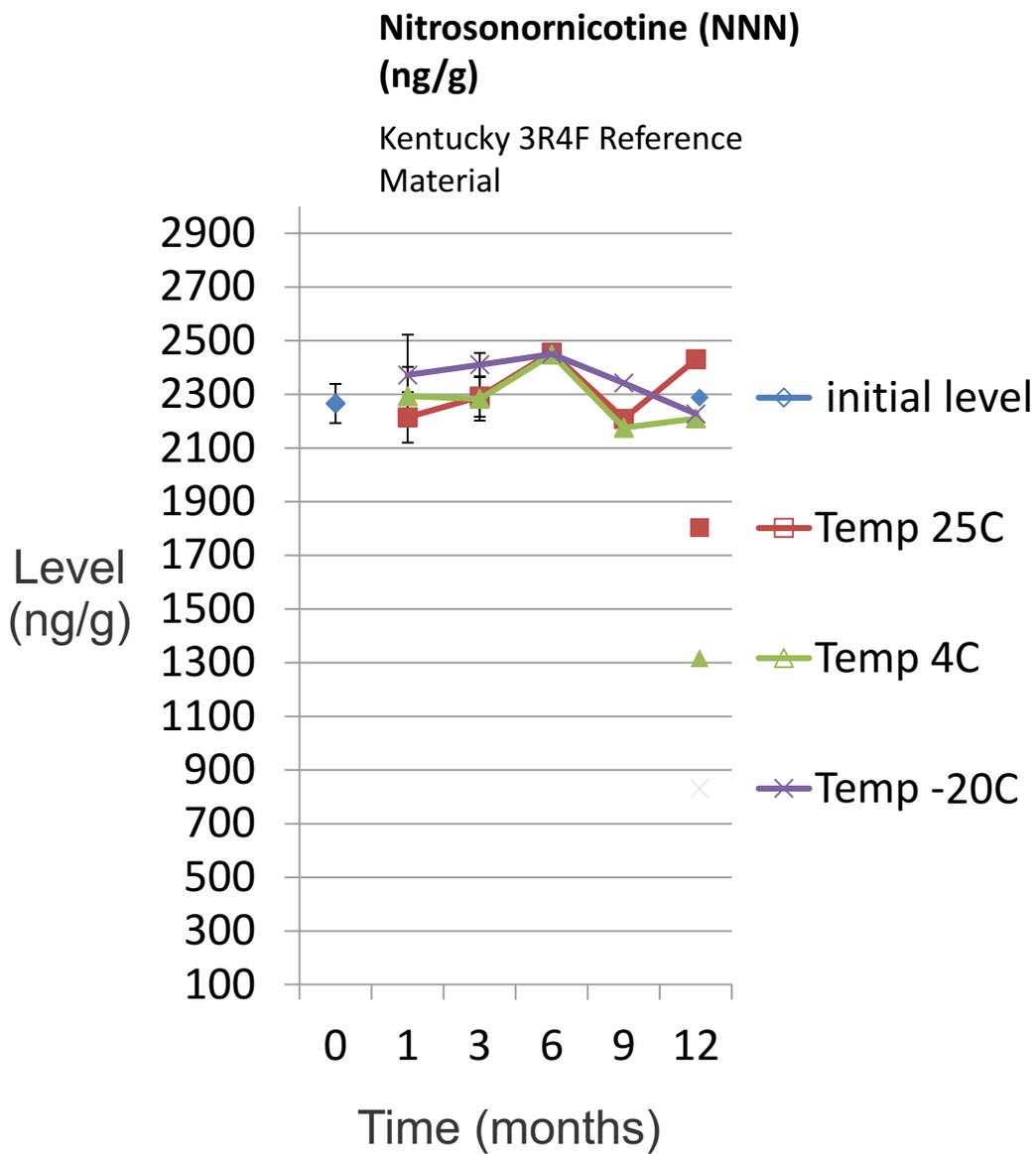


Figure S31. NNN levels (ng/g), plotted as a function of time (months), for Kentucky 3R4F Reference Material samples stored at different temperatures. Bars represent approximate 95% confidence intervals for 7 replicate measurements.

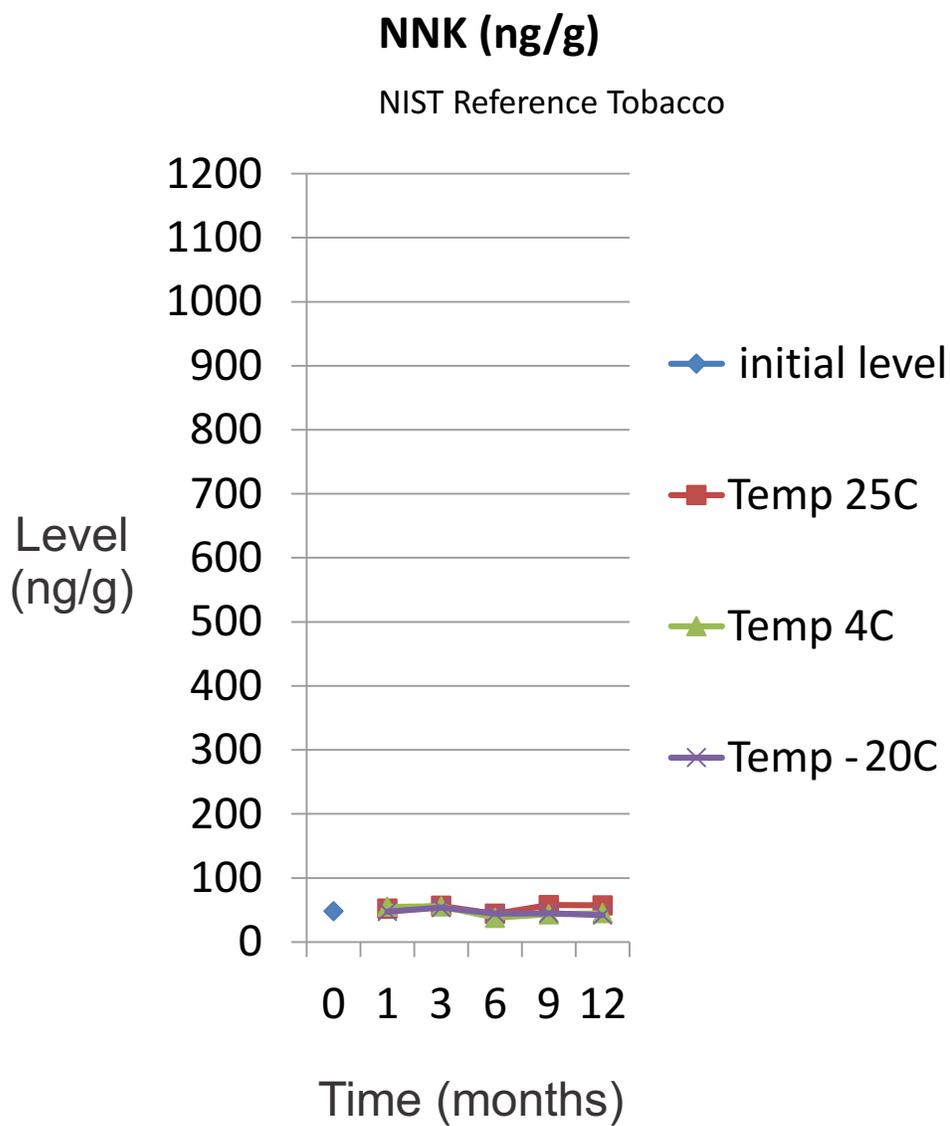


Figure S32. NNK levels (ng/g), plotted as a function of time (months), for SRM 3222 samples stored at different temperatures.

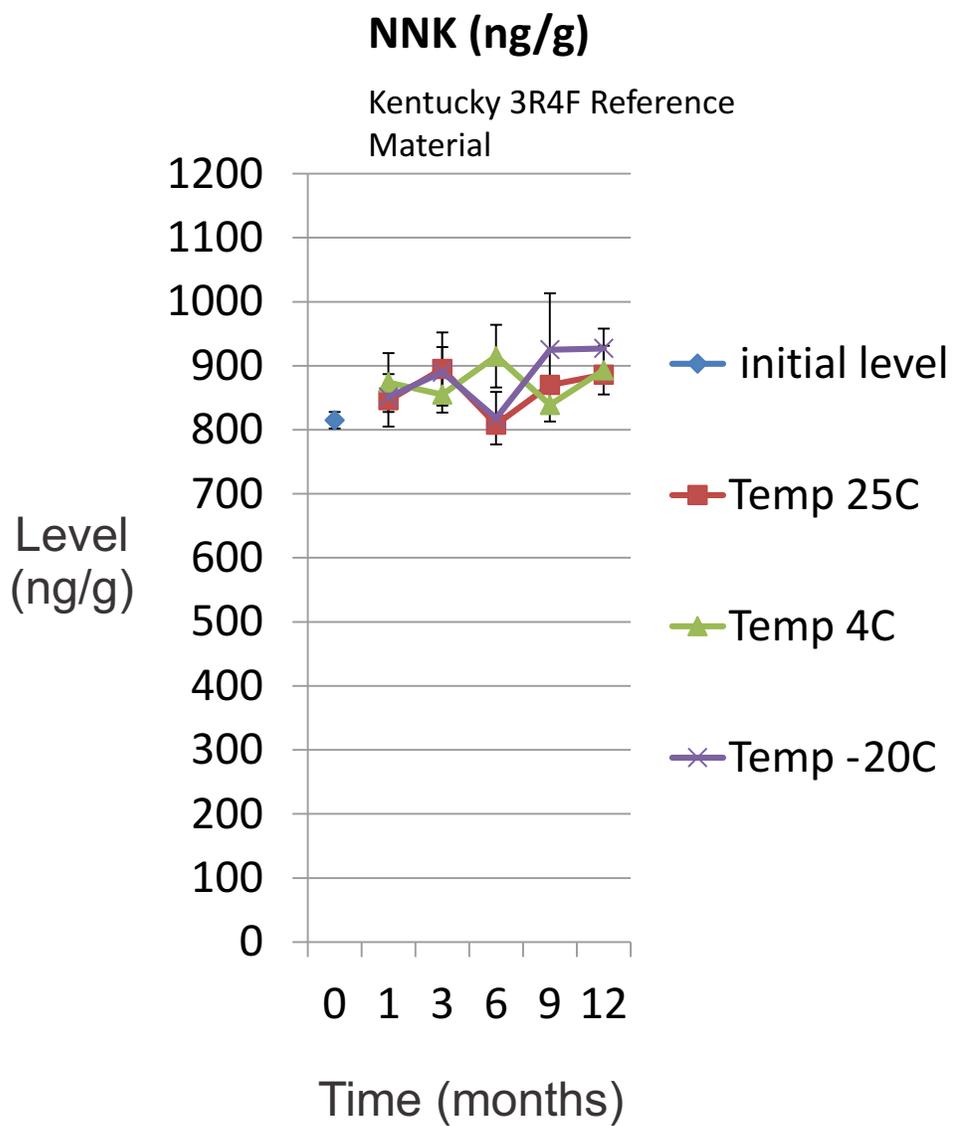


Figure S33. NNK levels (ng/g), plotted as a function of time (months), for Kentucky 3R4F Reference Material samples stored at different temperatures. Bars represent approximate 95% confidence intervals for 7 replicate measurements.

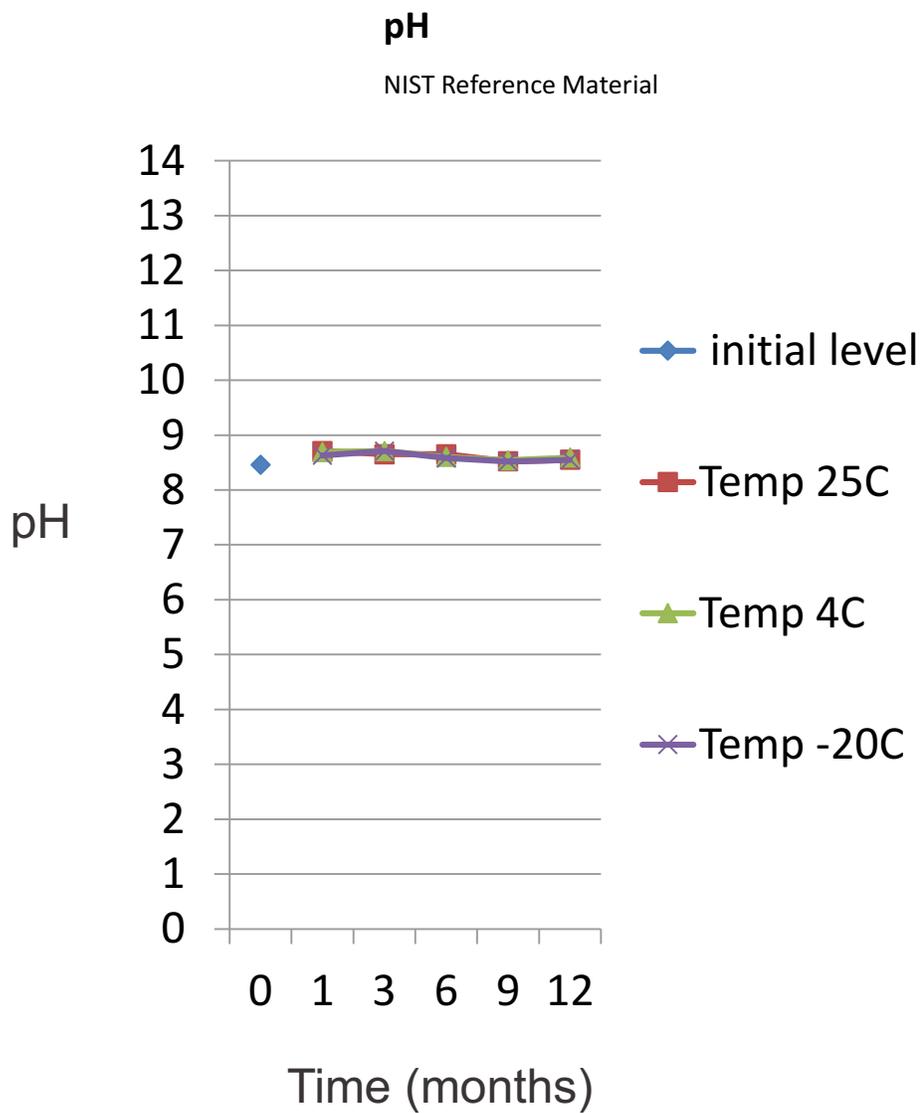


Figure S34. pH, plotted as a function of time (months), for SRM 3222 samples stored at different temperatures.

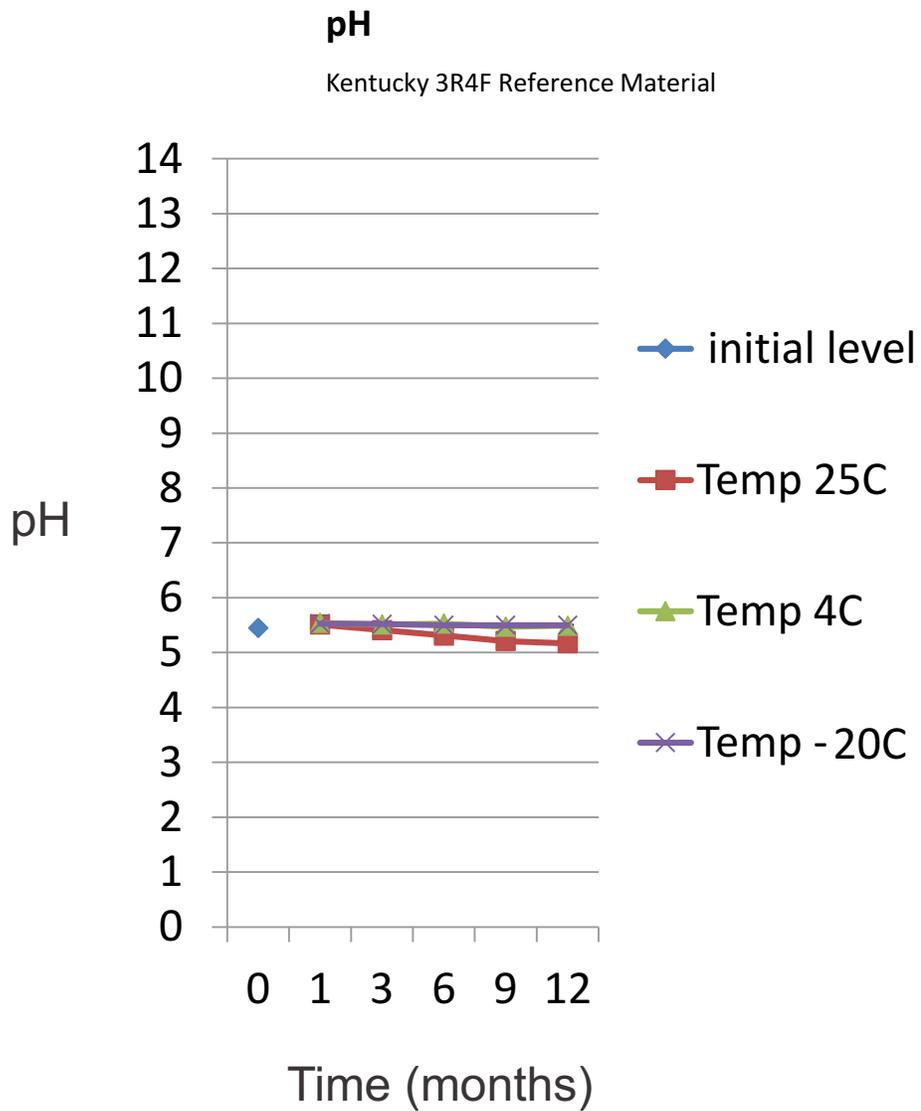


Figure S35. pH, plotted as a function of time (months), for Kentucky 3R4F Reference Material samples stored at different temperatures.

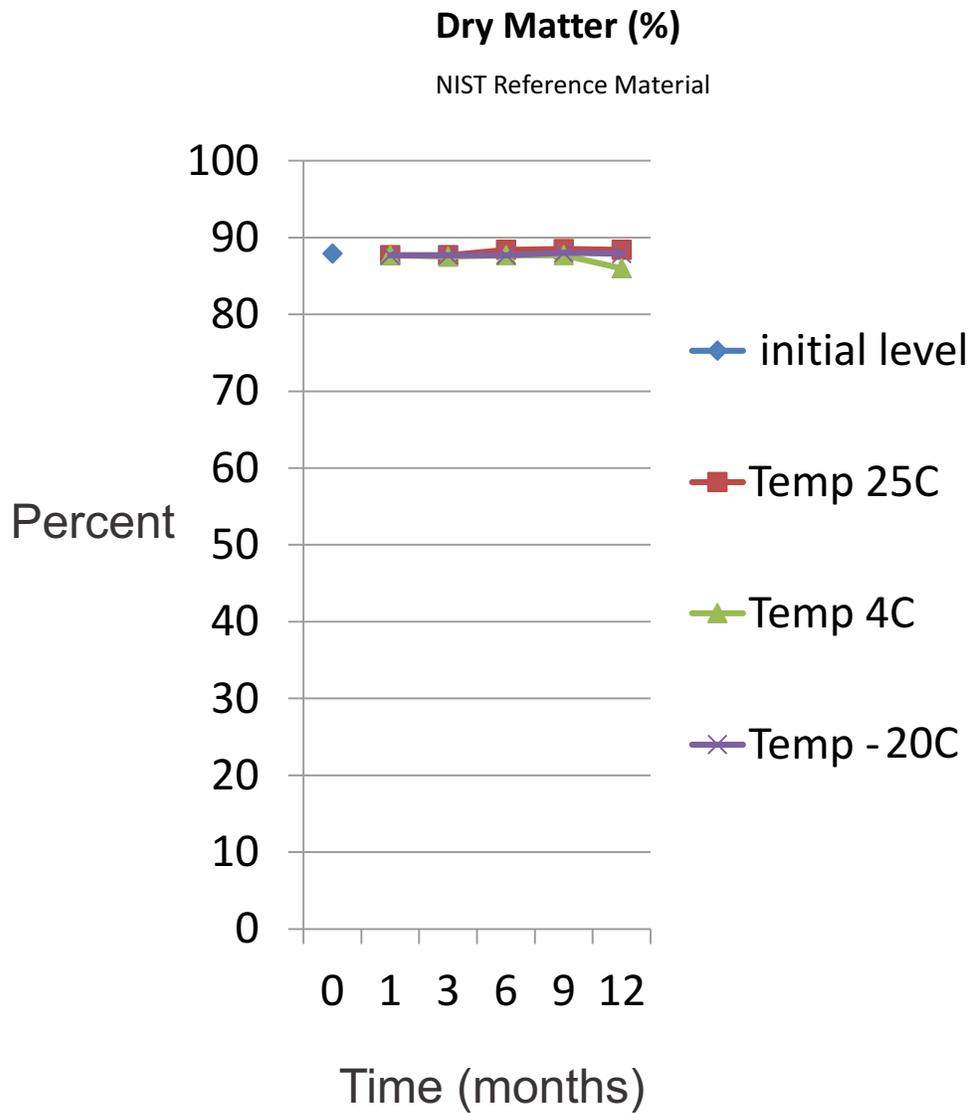


Figure S36. Dry matter (percent), plotted as a function of time (months), for SRM 3222 samples stored at different temperatures.

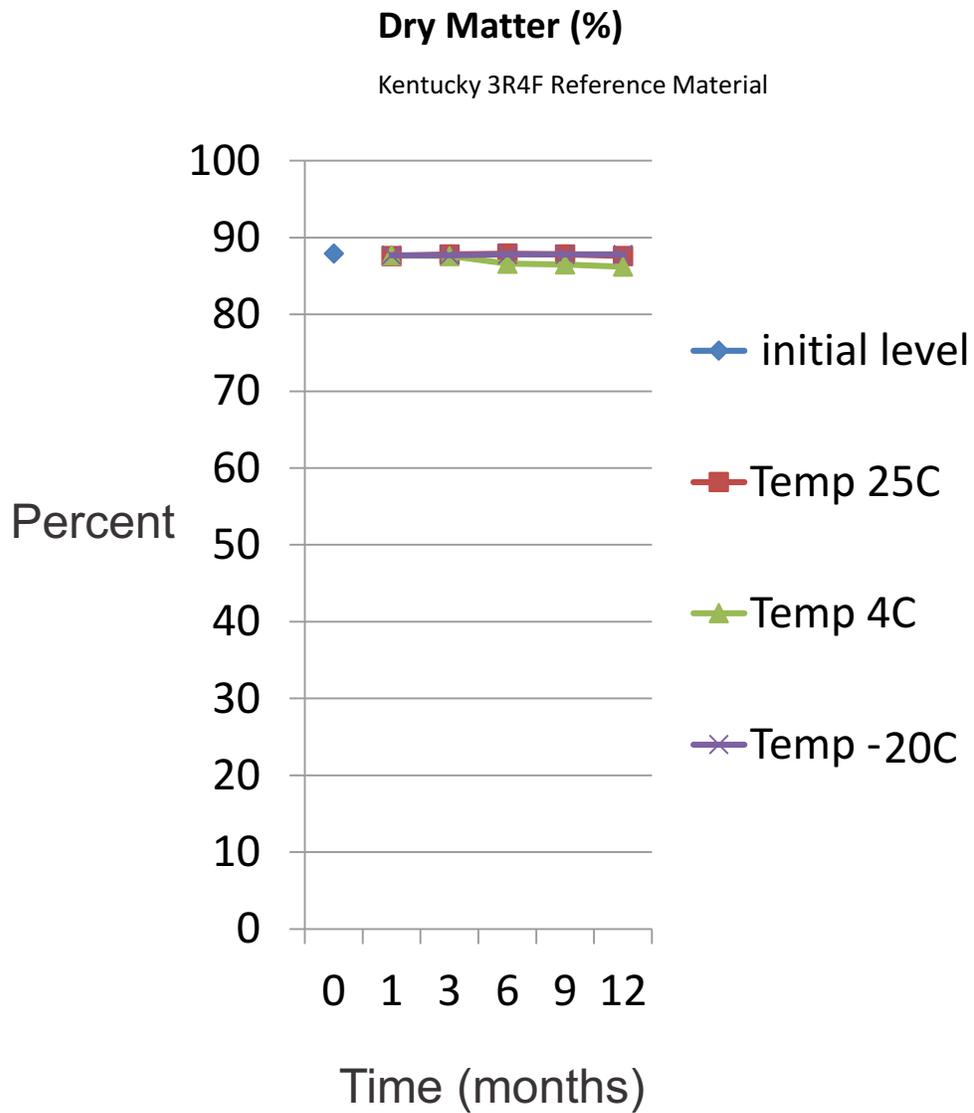


Figure S37. Dry matter (percent), plotted as a function of time (months), for Kentucky 3R4F Reference Material samples stored at different temperatures.

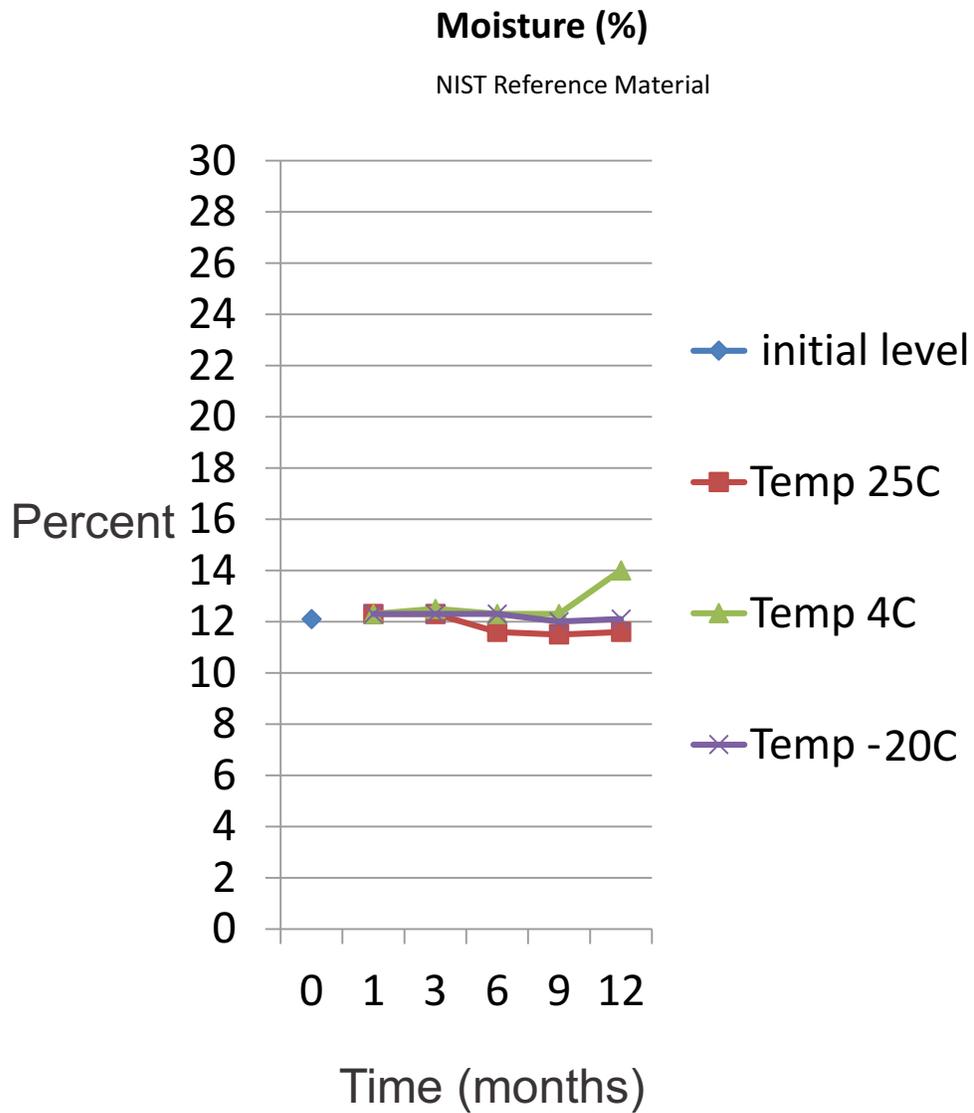


Figure S38. Moisture (percent), plotted as a function of time (months), for SRM 3222 samples stored at different temperatures.

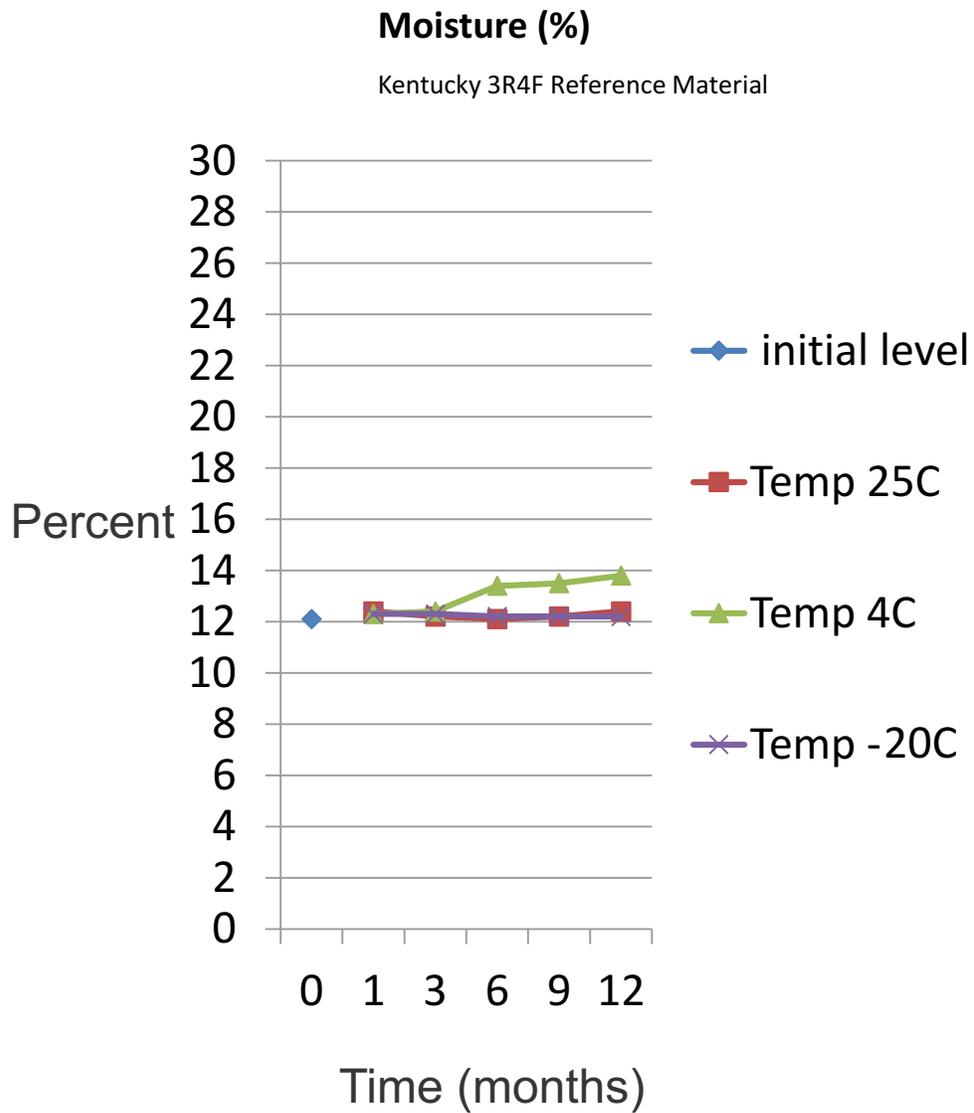


Figure S39. Moisture (percent), plotted as a function of time (months), for Kentucky 3R4F Reference Material samples stored at different temperatures.

### Freeze/Thaw Cycle: Nicotine (mg/g)

Kentucky 3R4F Reference Material  
NIST Reference Material

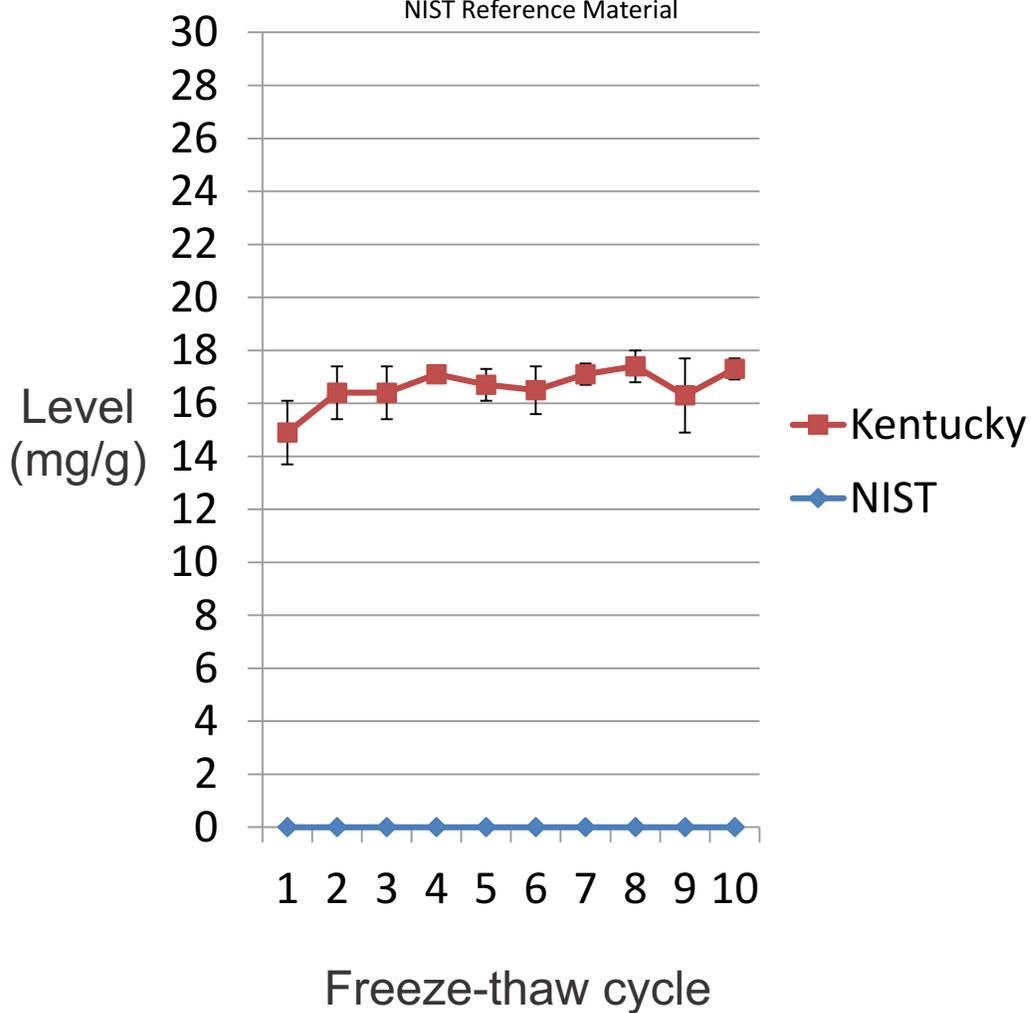


Figure S40. Nicotine levels (mg/g), plotted as a function of freeze-thaw cycle, for SRM 3222 and Kentucky 3R4F Reference Material samples. Nicotine levels in SRM 3222 were below detectable limits for this study (see text). Bars represent approximate 95% confidence intervals for 7 replicate

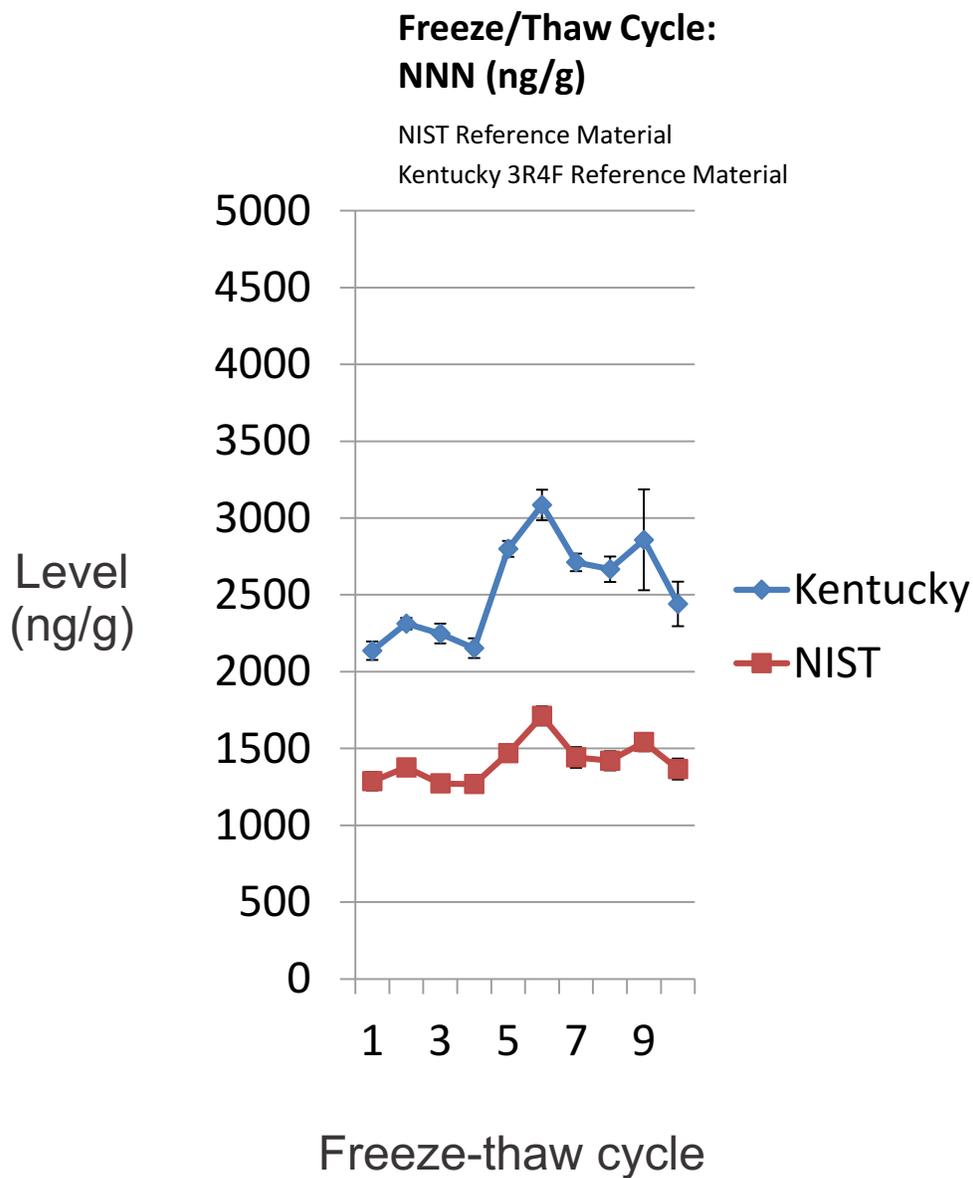


Figure S41. NNN levels (ng/g), plotted as a function of freeze-thaw cycle, for SRM 3222 and Kentucky 3R4F Reference Material samples. Bars represent approximate 95% confidence intervals for 7 replicate measurements.

**Freeze/Thaw Cycle:  
NNK (ng/g)**

NIST Reference Material  
Kentucky 3R4F Reference Material

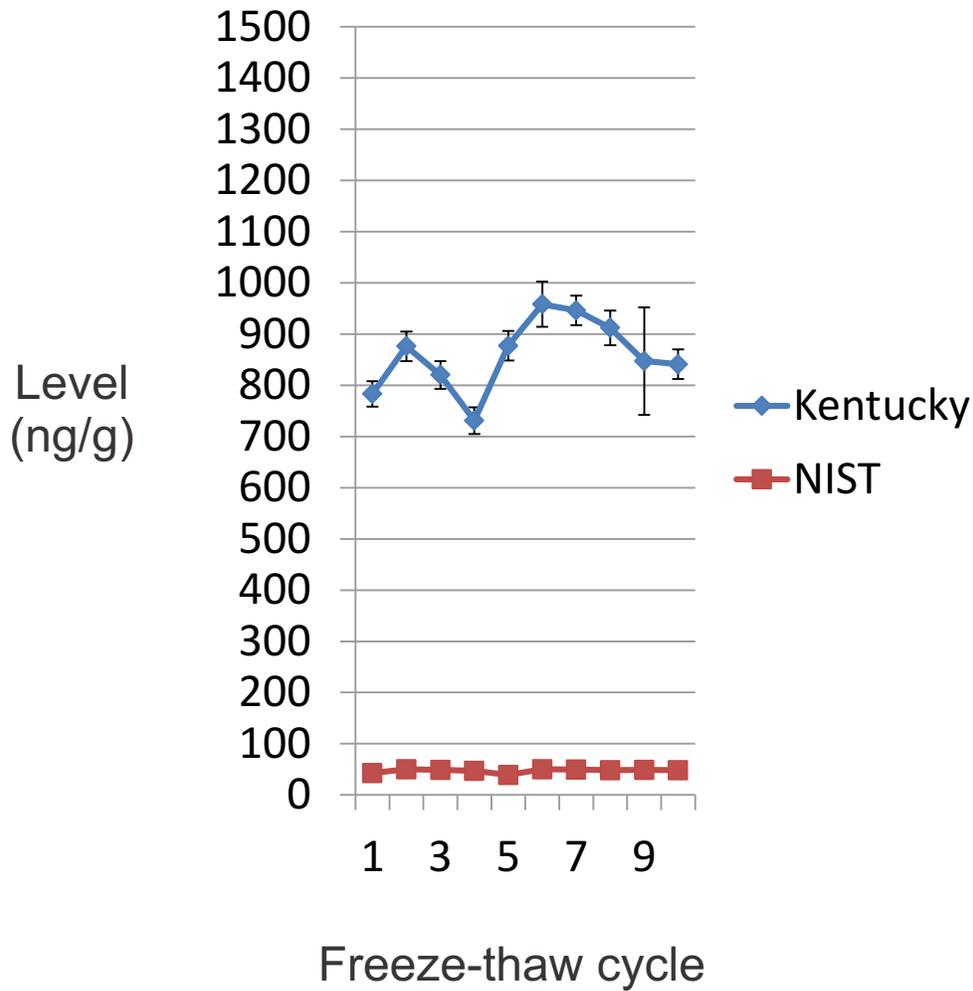


Figure S42. NNK levels (ng/g), plotted as a function of freeze-thaw cycle, for SRM 3222 and Kentucky 3R4F Reference Material samples. Bars represent approximate 95% confidence intervals for 7 replicate measurements.

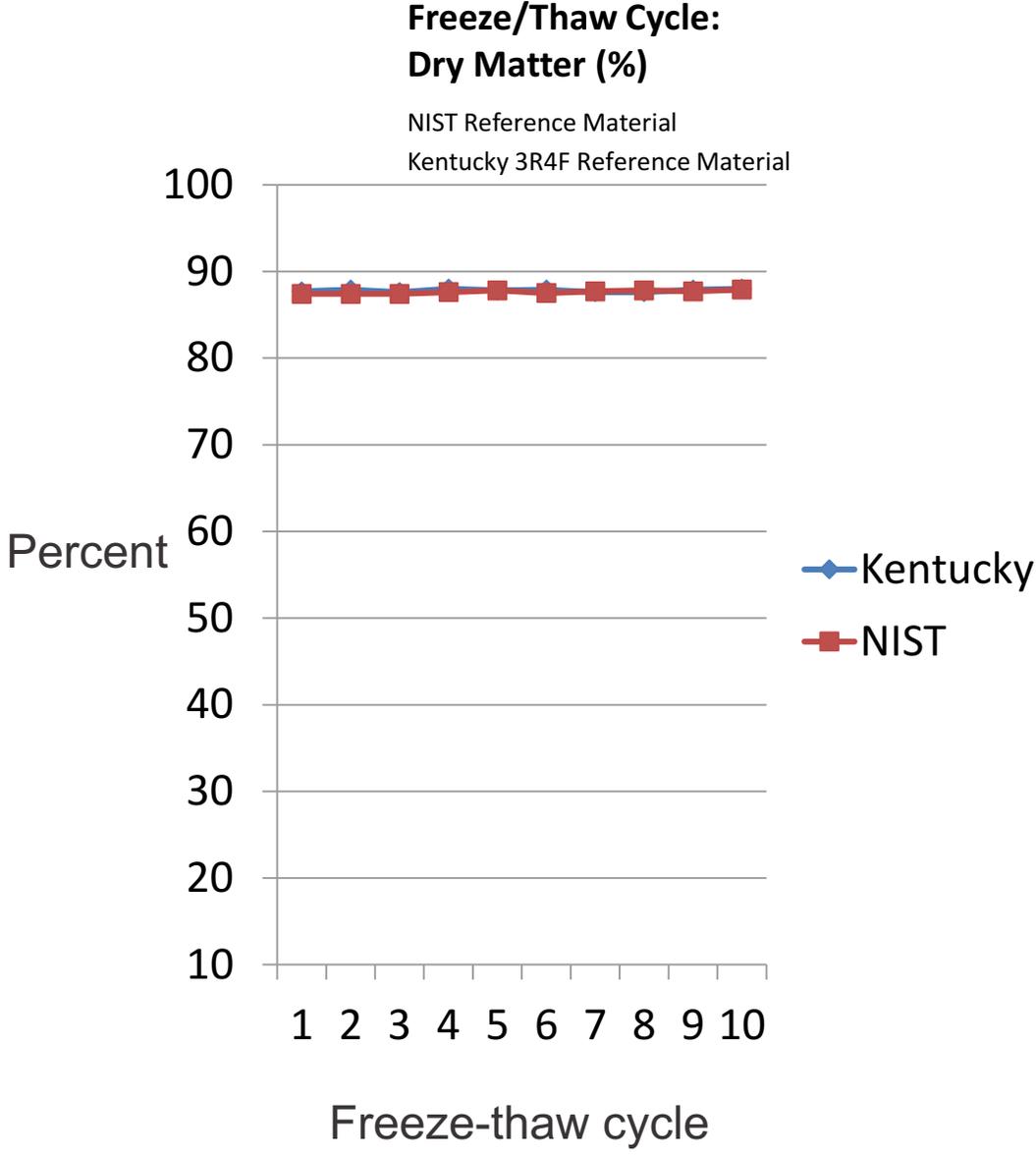


Figure S43. Dry Matter (percent), plotted as a function of freeze-thaw cycle, for SRM 3222 and Kentucky 3R4F Reference Material samples.

### Freeze/Thaw Cycle: Moisture (%)

NIST Reference Material

Kentucky 3R4F Reference Material

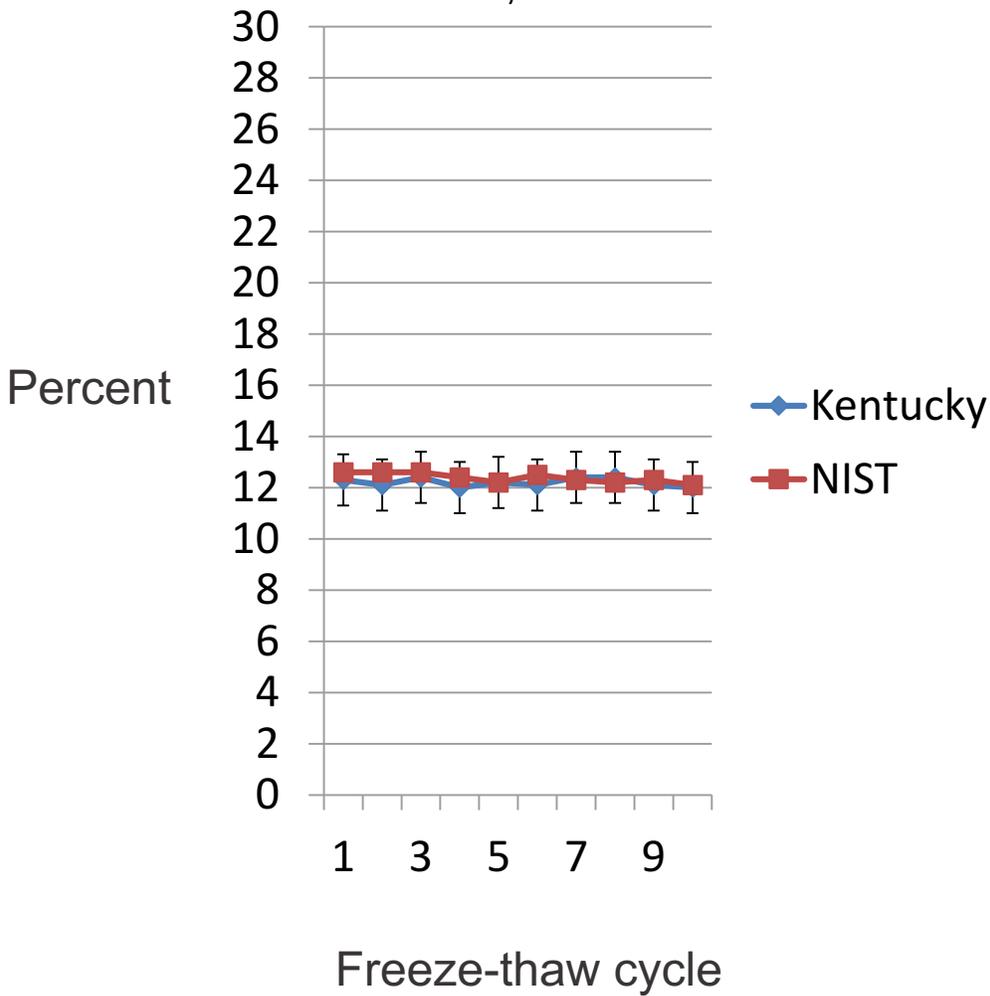


Figure S44. Moisture (percent), plotted as a function of freeze-thaw cycle, for SRM 3222 and Kentucky 3R4F Reference Material samples. Bars represent approximate 95% confidence intervals for 7 replicate measurements.

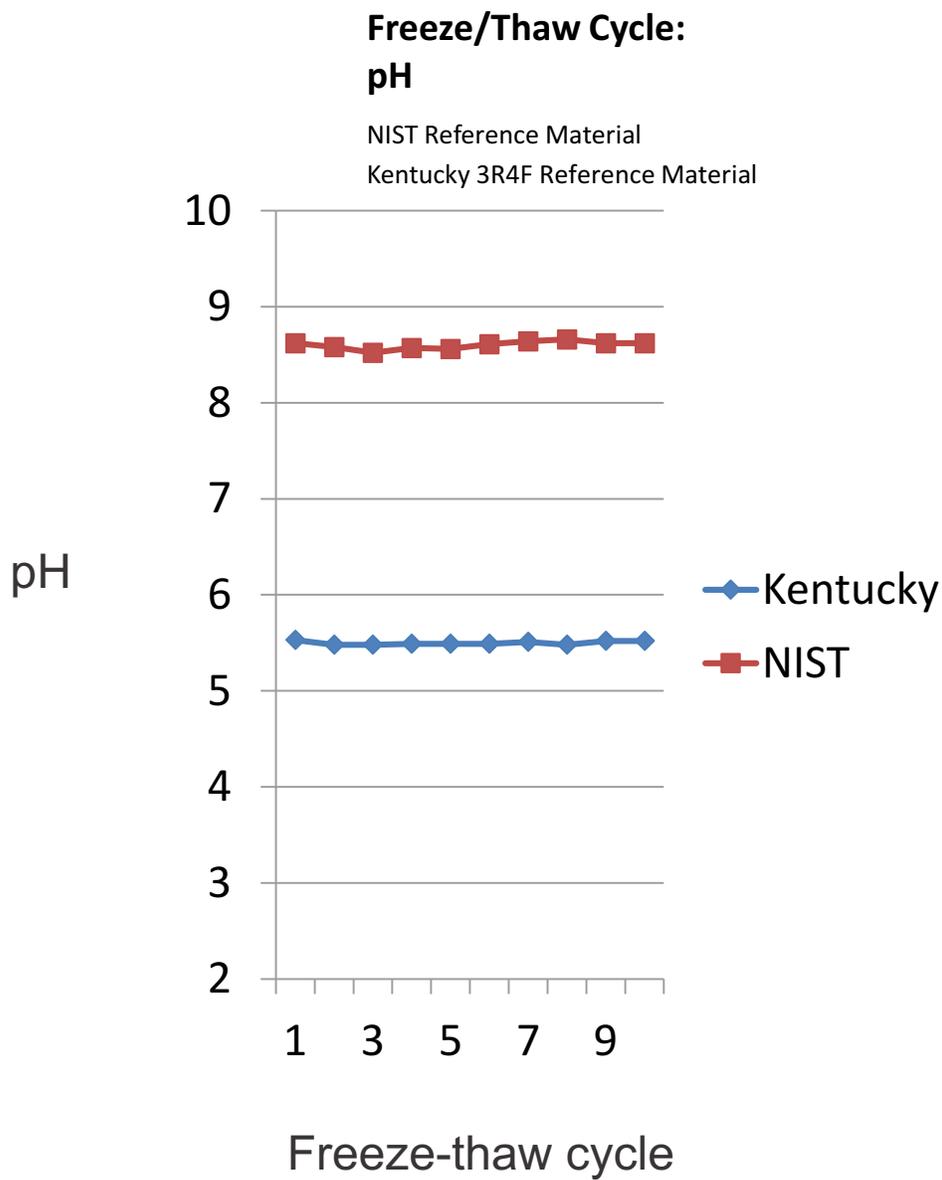


Figure S45. pH, plotted as a function of freeze-thaw cycle, for SRM 3222 and Kentucky 3R4F Reference Material samples.