TECHNICAL OPERATING PROCEDURE

PROCEDURE TITLE: Determination of Percent Active Ingredient of Technical Grade Manufacturing Use and End-use Bayluscide (Niclosamide, NIC) and 3-trifluoromethyl-4-Nitrophenol (TFM) in Lampricide Formulations

APPLICABILITY: Branch of Aquatic Ecosystem Health

PRINCIPLE: Percent active ingredient analysis is intended to ensure the concentration of an active ingredient in a sample. This will be achieved by comparison of a High-Performance Liquid Chromatography (HPLC) signal response to that of a reference standard. The active ingredient in all lampricides is either TFM or NIC.

PRECAUTIONS

A. Potential Interferences

1. Avoid chemical contamination. Take necessary precautions to minimize the possibility of contamination. Examples include use of properly cleaned glassware (SOP No. AEH 008) and use of HPLC-grade solvents.

B. Safety

1. Standard laboratory safety apparel (lab coats, gloves, and safety glasses) should be worn when handling solvents and chemicals when conducting the described procedure.

2. Follow the Safety Data Sheets for chemicals used in this procedure.

PROCEDURE

A. Reagents

1. Water, HPLC grade or equivalent (such as e-pure)
2. Methanol, HPLC grade or better
3. Ammonium Acetate (ACS Reagent grade, ≥98% pure)
4. Acetic Acid, glacial (ACS Reagent grade, ≥99% pure)
5. 3-trifluoromethyl-4-Nitrophenol, CAS # 88-30-2 (Reagent grade, ≥99% pure)
6. Niclosamide, CAS # 50-65-7 (Reagent grade, ≥99% pure)

B. Apparatus

1. Analytical Balance (five-place)

2. Spatulas: Stainless steel, 23 mm.

3. Volumetric Flasks: Class A, 10–1,000 mL.

4. Volumetric Pipettes: Class A, 1–10 mL.

5. HPLC Sample Vials: Amber or clear glass with PTFE/Silicone septa.

6. HPLC System: Agilent 1100 Series or Agilent 1260 Series HPLC composed of a degasser, binary pumping system, autosampler, column heater, diode array detector and Agilent Chemstation (or OpenLab) software for system control and UV data reduction or equivalent HPLC system. Refer to Agilent's 1260 User Guides for instrument operation instructions and Agilent Chemstation Guides (or OpenLab Guides) for software operation instructions. Follow SOP No. AEH 237 for general HPLC use.

C. Procedure for Preparation of Standards and Solutions

1. Stock solution of TFM or NIC: Weigh 0.0100 g of analytical-grade chemical on a five-place analytical balance. Record the weight of chemical to five decimal places. Dissolve chemical in a 100 mL volumetric flask with methanol (HPLC grade or equivalent) for a 100 mg/L nominal standard.

2. TFM Calibration Standards: Dilute the stock solution with water to desired concentrations which bracket the expected concentration in the samples (i.e., 105 mg/L x 10mL / 100 mL = 10.5 mg/L Standard). A minimum of five standards are desired. Nominal concentrations of 5, 4, 3, 2, and 1 mg/L are suggested for TFM Bar assays. Nominal concentrations of 15, 10, 7, 4, and 1 mg/L are suggested for TFM Field Standard assays.

3. Water:Methanol (40:60) Solution: Transfer 400 mL methanol from a graduated cylinder to a 1 L volumetric flask. Fill the flask with water and mix. Cool the flask to room temperature and fill the flask with water again. Other amounts of this solution may be made.

4. Niclosamide Calibration Standards: Dilute the stock solution with water:methanol (40:60) to desired concentrations which bracket the expected concentration in the samples. A minimum of five standards are desired. Nominal concentrations of 2.00, 1.00, 0.50, 0.20, and 0.10 mg/L are suggested for all NIC assays.

5. Mobile Phase A: 10 mM ammonium acetate buffer (pH 4.0) in water:methanol (75:25)

   a. Weigh 0.770 g of ammonium acetate.

   b. Transfer the ammonium acetate to a 1 L volumetric flask and fill half way with e-pure water.

   c. Add 3 mL glacial acetic acid. Add 250 mL methanol and mix. Cool the flask to room temperature. Fill the flask to volume with e-pure water and mix.

   d. Mobile Phase A expires seven days from the date made.

6. Mobile Phase B: 10 mM ammonium acetate buffer in methanol

    a. Weigh 0.770 g of ammonium acetate.

    b. Transfer the ammonium acetate to a 1 L volumetric flask and fill half way with methanol.

    c. Add 3 mL glacial acetic acid. Fill the flask to volume with methanol and mix.

    d. Mobile Phase B expires fourteen days from the date made.

D. Preparation of Test Article for % Active Ingredient Analysis

1. Ensure that the test article is uniformly mixed, especially liquid samples, by shaking the sample prior to sub-sample preparation. Prepare one batch in triplicate to assess batch variability.

2. TFM Bar: Weigh out 0.500 g on a five-place balance. Record the weight to five decimal places. Transfer the bar to a 500 mL volumetric flask, bring to volume with methanol and mix. Sonicate until chemical is dissolved, mix, and allow the solution to cool to room temperature. Verify the flask is filled to volume. If not, add more methanol to reach volume and mix. Transfer 1.0 mL to a 100 mL volumetric flask, bring the flask to volume with water, and mix. Transfer about 1 mL into labeled LC vial with Pasteur pipette. To calculate the %TFM: Percent TFM = HPLC Concentration (mg/L) x Dilution factor (50) /Sample weight (mg) x 100 (%).

3. TFM Field Standard: Pipette the field standard directly into a labeled LC vial. The TFM concentration to report is given in the LC output. No further calculations are required.

4. NIC Field Standard: Transfer 1.0 mL of field standard into a 100 mL volumetric flask, fill the flask to volume with water:methanol (40:60), and mix. Transfer about 1 mL into labeled LC vial with Pasteur pipette. To calculate the NIC concentration: NIC (mg/L) = HPLC concentration (mg/L) x Dilution factor (100).

5. Bayluscide Granules: Weigh out 0.100 g on a five-place balance. Record the weight to five decimal places. Transfer to a 100 mL volumetric flask, bring to volume with methanol and mix. Sonicate until chemical is dissolved off the granules, mix, and allow the solution to cool to room temperature. Verify the flask is filled to volume. If not, add more methanol to reach volume and mix. Transfer 2.0 mL to a 100 mL volumetric flask, bring the flask to volume with water:methanol (40:60), and mix. Transfer about 1 mL into labeled LC vial with Pasteur pipette. To calculate the %NIC: Percent NIC = HPLC Concentration (mg/L) x Dilution factor (5) /Sample weight (mg) x 100 (%). Multiply percent NIC by the conversion factor of 1.186 to obtain percent bayluscide.

6. Bayluscide Technical: Weigh out 0.010 g on a five-place balance. Record the weight to five decimal places. Transfer to a 100 mL volumetric flask, bring to volume with methanol and mix. Sonicate until chemical is dissolved, mix, and allow the solution to cool to room temperature. Verify the flask is filled to volume. If not, add more methanol to reach volume and mix. Transfer 1.0 mL to a 100 mL volumetric flask, bring the flask to volume with water:methanol

(40:60), and mix. Transfer about 1 mL into labeled LC vial with Pasteur pipette. To calculate the %NIC: Percent NIC = HPLC Concentration (mg/L) x Dilution factor (10) /Sample weight (mg) x 100 (%). Multiply percent NIC by the conversion factor of 1.186 to obtain percent bayluscide.

7. Bayluscide 20% Emulsifiable Concentrate: Weigh out 0.100 g on a five-place balance. Record the weight to five decimal places. Transfer to a 100 mL volumetric flask, bring to volume with methanol and mix. Sonicate until chemical is dissolved, mix, and allow the solution to cool to room temperature. Verify the flask is filled to volume. If not, add more methanol to reach volume and mix. Transfer 1.0 mL to a 200 mL volumetric flask, bring the flask to volume with water:methanol (40:60), and mix. Transfer about 1 mL into labeled LC vial with Pasteur pipette. To calculate the %NIC: Percent NIC = HPLC Concentration (mg/L) x Dilution factor (20) /Sample weight (mg) x 100(%). Multiply percent NIC by the conversion factor of 1.186 to obtain percent bayluscide.

8. Bayluscide 70% Wettable Powder: Weigh out 0.010 g on a five-place balance. Record the weight to five decimal places. Transfer to a 100 mL volumetric flask, bring to volume with methanol and mix. Sonicate until chemical is dissolved, mix, and allow the solution to cool to room temperature. Verify the flask is filled to volume. If not, add more methanol to reach volume and mix. Transfer 1.0 mL to a 100 mL volumetric flask, bring the flask to volume with water:methanol (40:60), and mix. Transfer about 1 mL into labeled LC vial with Pasteur pipette. To calculate the %NIC: Percent NIC = HPLC Concentration (mg/L) x Dilution factor (10) /Sample weight (mg) x 100(%). Multiply percent NIC by the conversion factor of 1.186 to obtain percent bayluscide.

E. Analysis of Test Chemical for Percent Active Ingredient

1. Set and confirm HPLC method parameters.

   a. Column: Kinetex XB-C18, 2.6 µm, 100 Å, 3.0 x 50 mm, (Phenomenex, Torrance, CA) or equivalent.

   b. In-Line Filter: KrudKatcher ultra, 0.5 um depth filter x 0.004 in. ID or equivalent.

   c. Guard Column (optional): SecurityGuard Ultra with C18 cartridge #AJO-8775 or equivalent.

   d. Column Temperature: 50°C.

   e. Injection Volume: 1-50 µL. Normally 25 µL for TFM and 25 µL for NIC.

   f. Mobile Phase conditions for TFM: Flow rate is 1.25 mL/ minute. Initial gradient condition is 25% B. At time 0.00 min. switch to 40% B. Maintain 40% B until 0.50 min. At time 0.50 min. start descending to reach 25% B at 0.60 min.

   g. Mobile Phase conditions for NIC: Flow rate is 1.25 mL/ minute. Initial gradient condition is 55% B. At time 0.00 min. switch to 70% B. Maintain 70% B until 0.50 min. At time 0.50 min. start descending to reach 55% B at 0.60 min.

h. Detector wavelength settings for TFM: The signal for TFM is 295 nm with a bandwidth of 10 and reference signal of 350 nm with a bandwidth of 80 nm.

i. Detector wavelength settings for NIC: The signal for NIC is 336 nm with a bandwidth of 8 and reference signal of 370 nm with a bandwidth of 40 nm.

j. Detector Spectra for TFM and NIC: Store spectra from 200-400 nm at apex, slope and baseline of peaks.

2. Equilibrate the HPLC system. Determine system suitability of the HPLC system according to defined procedures outlined in SOP No. AEH 237.

3. Place the calibration standards and samples in the HPLC autosampler.

4. Prepare a sample set (Sequence) in the following order

   a. Full set of calibration standards from lowest to highest

   b. Matrix Blank

   c. Samples prepared in step D

      (1.) Mid-range QC standard after every ten sample injections

   d. Matrix Blank

   e. Full set of calibration standards from lowest to highest

   f. Matrix Blank

      Note: It is acceptable to insert additional standards between samples for sequences that contain a large number of samples.

5. Verify that the system is stable (less than 2% change in retention time and peak area from consecutive injections of the same standard). Note: Retention and peak areas may vary from sequence to sequence. The capacity factor (K') should be between 3 and 5 for all injections (K'= (TR-T0)/T0).

6. Start the sequence.

7. Review the data and generate a report.

   a. The standard curve is acceptable if the correlation coefficient is ≥0.995. If the correlation is <0.995, prepare new standards.

   b. The retention times are acceptable if the K' is between 3 and 5 and does not vary by more than ±5%.

c. The QC standard checks must be within ± 10% of the theoretical concentration. If the QC standard check falls outside these criteria, samples must be reanalyzed.

d. Peak symmetry should be greater than 0.55 for all peaks. If the peak symmetry is ≤0.55 samples must be reanalyzed.

e. Calculate the mean, SD, and %RSD of replicate injections. If %RSD is >15% the sample should be re-analyzed (AEH SOP 407). Use this mean to calculate the % active ingredient or concentration for each subsample analyzed.

f. Calculate the mean of replicate subsamples of a batch (n=3), SD, and %RSD. If %RSD is >20%, the subsamples should be re-analyzed (AEH SOP 407). Report the mean of the subsamples as the % active ingredient or concentration.

REFERENCES

A. 1260 User's Guides for instrument operation instructions

B. Agilent Chemstation Guides.

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Change History Table

Revision No.	Date	Person Responsible	Description of Changes
05	04/23/19	Justin Schueller, Nicholas Schloesser and Kim Fredricks	Deleted material in B2 safety, deleted specific balance in B1 Apparatus, updated user guide to 1260, added hyphens between five and place throughout, spelled out niclosamide in C4, removed verbage in parentheses in E4a–f. Updated SOP to reflect QMS guidance of April 20, 2018, which included updates to the header, addition of a footer, and inclusion of change history table.