

IOP 015.5  
Effective Date: 1/09/2017  
Replaces IOP 015.4  
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U.S. Fish and Wildlife Service  
Marquette Biological Station  
3090 Wright Street  
Marquette, Michigan 49855  
U.S.A.

and

U.S. Fish and Wildlife Service  
Ludington Biological Station  
5050 Commerce Drive  
Ludington, Michigan 49431  
U.S.A.

and

Department of Fisheries and Oceans  
Sea Lamprey Control Centre  
1219 Queen Street East  
Sault Ste. Marie, Ontario P6A 2E5  
Canada

## **INSTRUMENT OPERATING PROCEDURE**

### **INSTRUMENT:**

High Performance Liquid Chromatograph with Empower III Software

### **MODEL:**

Detector; model 2489

Pump; model 515

Injector; Rheodyne 7725i

Laptop computer running Empower III Software

Temperature Control Module; model TCM or TCM 2

Column Heater Module; model CHM

### **MANUFACTURER:**

Waters Corporation

### **SAFETY:**

Before operation of instrument read all MSDS for reagents used in procedures. Follow standard and accepted laboratory safety guidelines.

## PROCEDURES:

- I. Set-up of Waters high performance liquid chromatograph (HPLC)
  - A. Instrument components
    1. The high performance liquid chromatograph components are modular to facilitate transport and replacement.
    2. The modules include:
      - a. Temperature control system including:
        - (1) Temperature control module (I or II)
        - (2) Column heater module containing column
      - b. Model 515 HPLC pump
      - c. Model 2489 tunable absorbance detector
      - d. Laptop computer
      - e. Rheodyne 7725i injector
  - B. Instrument placement, power requirements, and connections
    1. Place the instrument modules on a counter top away from drafts, vibrations, and direct sunlight.
    2. During transport, the pathway of stainless steel tubing for the carrier solution is broken at three sites. Each connection is reestablished:
      - a. At the union between the pump and injector
      - b. At the pre-column between the injector and column heater
      - c. At the port on the face of the detector
    3. Five of the six primary modules require an AC power outlet. A power strip connected to a line-power conditioner is preferred.
    4. Connect the signal cables to the model 2489 detector
      - a. The cable from the back of the column heater is connected to the temperature control module at the port labelled UNIT 1 (CHM 1 for the TCM 2).
      - b. The cable from the back of the injector is connected to the detector port II with the 10 pronged I/O connector.
      - c. The detector is connected to the laptop computer with a **crosslink** cable. The cable plugs into the data port on the detector and is connected to the laptop via the ethernet connection.
    5. Replace the pre-column insert.
  - C. Temperature control system
    1. This system reduces drift in retention time due to fluctuations in ambient temperature by maintaining a constant temperature for the chromatography column.
    2. Column heater
      - a. Turn the **POWER** on.
      - b. Allow at least 30 minutes for the temperature of the unit to stabilize before making injections. The TCM may indicate that the temperature has been reached sooner, but the column may not be up to temperature.

3. Temperature Control Module
  - a. Turn **POWER** on.
  - b. Enter the desired temperature for the column:
    - (1) Press **SET**.
    - (2) Enter 350 and **ENTER**. This sets the operating temperature at 35.0 °C. If the setting is not entered immediately the display will revert to the temperature of the column heater.
  - c. Enter an upper temperature limit:
    - (1) Press **OVER**
    - (2) Enter 500 and **ENTER**. This sets the maximum temperature limit at 50.0 °C. This parameter prevents overheating of the column.
  
4. Temperature Control Module 2
  - a. Ensure the column heater is plugged into port CHM 1.
  - b. Turn the **POWER** on.
  - c. Press the **MENU** button.
  - d. Select temperature settings. Press **ENTER**.
  - e. Select column heater module 1. Press **ENTER**.
  - f. Using the arrows to toggle through the screen:
    - (1) Set the upper limit to 50°C. This prevents overheating of the column.
    - (2) Set the set point to 35°C.
    - (3) Set the lower limit to 34°C.

Note: If the lower limit is the same as the set point an error message will occur. Ensure that the lower limit is 1°C lower than the set point value.
  - g. Press **HOME**.
  - h. Turn on the column heater

D. Waters model 515 pump

1. Formulate carrier solution according to instructions in section V, and place intake tube into the carrier solution (it is preferable to formulate the carrier solution the day before use).
2. Prime the pump:
  - a. Fully depress the priming syringe plunger to remove all air from the syringe, then insert the syringe into the luer fitting at the center of the draw-off valve handle.
  - b. Turn the draw-off valve handle counterclockwise about 1/2-turn to open the valve.
  - c. Withdraw several milliliters of eluent with the syringe, then close the valve handle.
  - d. Expel the contents of the syringe into a suitable waste container.
3. Turn **POWER** on. When FLOW MENU appears, press **EDIT/ENTER** button. The flashing cursor will appear.
4. The **EDIT/ENTER** button controls the position of the cursor. Change the display from 1.000 to 0.500 and press the **MENU** button to enter the value.
5. Press the **RUN/STOP** button to start the pump. The flow rate displayed on the

bottom line will show 0.500 mL and a pressure reading will be displayed.

6. Change the flow to 0.700 and enter the value with the **MENU** button. When **MENU** is pressed the flow will change to 0.700 mL. Slowly continue to raise the flow rate in 0.2 or 0.3 mL increments with the **UP** and **DOWN** arrows and the **MENU** button until the flow is 2.000 mL for niclosamide/1.0 mL for TFM.
- E. Waters model 2489 uv/visible detector
1. Turn detector **POWER** on.
  2. The detector will require several minutes to conduct a series of self checks on systems and calibrations on the light source. The detector is ready to accept commands when **<idle mode>**, **WAVELENGTH** and **AUFS** are displayed. If the lamp fails to light during the start-up period shut down the detector and restart. If the detector is started before the internal flow cell is filled with carrier solution, the self checks may fail – if this occurs restart the detector after carrier is coming out of the waste tube.
  3. Confirm that the detector sensitivity is set at 2:
    - a. Use the up and down arrows to select **AUFS**.
    - b. A sensitivity of 2 will be displayed.
  4. Set the operating wavelength:
    - a. Use the up and down arrows to select the wavelength.
    - b. Enter the appropriate wavelength:
      - (1) Niclosamide: 330 and **ENTER**.
      - (2) TFM: 295 and **ENTER**.
- F. Start laptop computer running Empower III software
1. Turn laptop computer **POWER** on. Log in using windows username and password.
  2. Open Empower III software and enter the **USER** name and **PASSWORD**.
  3. Ensure the proper detector is online otherwise an error message will occur:
    - a. Select **CONFIGURE SYSTEM**.
    - b. Select system.
    - c. Right click on the desired detector.
    - d. Select **BRING ONLINE**.
    - e. Note: only one detector can be online at one time. If the desired detector is not online you must bring any other online systems offline by right clicking and selecting **BRING OFFLINE**.
- G. Creating New Project
1. Enter user name and password - system, manager
  2. Click on Configure System
  3. Click on File – New – Project
  4. Select Project to nest under – click next

5. Set table space to 200 MB, disable Full Audit Trail, default algorithm = Apex Track – click next
6. Disable Photo Diode Array (PDA) - click next
7. Keep as Owner Only on Access Control – click next
8. Copy methods and preferences from Template project
9. Enter project name
10. Click Finish to create project

H. New Instrument Method and Method Set creation

1. Open Run Samples and select Project and Chromatographic System (e.g. MBS HPLC 1)
2. Click on Develop Methods
3. Click on desired Instrument method or click new for a new method
4. In instrument editor ensure that entries are 60 Hz, Channel 1 = On, sampling rate to 5 Hz and that proper wavelength is selected.
5. Click File – Save as – Enter name
6. Click next on Method set window
7. Select applicable processing, report and export methods
8. Save method set name, click finish

I. Making an Injection

1. Open Run Samples and select Project and Chromatographic System (e.g. MBS HPLC 1)
2. Enter Sample or Standard name
3. Set Function to either Inject Samples or Inject Standards
4. Enter desired Method Set
5. Ignore Vial and Injection Volume
6. Enter desired Run Time
7. Press Inject button and load sample (**INJECTOR MUST BE IN LOAD POSITION BEFORE PRESSING BUTTON**)
8. Turn injection valve to inject when Waiting for Injection is displayed in status bar at bottom of window.

J. Processing Method Creation

1. Select Browse Project, choose Project
2. In the Channels tab open a chromatogram from a standard injection
3. Click on Processing Wizard button, click next
4. Select Create a New Processing Method, click next
5. Ensure processing type is LC and algorithms is Apex Track. Ensure Use Processing Method Wizard is selected. Ensure Cross Channel Internal Standard is not selected, click next
6. Highlight desired Integration time, click next
7. Keep default Peak Width and Threshold (do not clear them), click next
8. Select Minimum Area (recommended 200), click next
9. Quantification should be **Area**, Component is **Amount** and Calibration Fit is **Linear**, click next
10. Select **No** to Cross Channel Processing, click next
11. Select desired peak and enter a name for component (Niclosamide), click next
12. Enter standard concentration and units (50 and ppb), click next
13. Select External Standard Calibration, click next
14. Enter Processing Method name, click finish.

K. Increase Retention Time Window in Processing Method

1. Select Browse Project, choose Project
2. Open Methods tab
3. Open Processing Method
4. Click on Components tab
5. Change RT Window (%) to 15
6. To update retention times – click on Update RT in same window, select type of update required (average retention times?)

L. New Method Set

1. Select Browse Project, choose Project

2. Click File – New method – New Method Set
3. No to Wizard
4. Select desired Instrument Method
5. Select desired Processing Method
6. Select any desired Report and Export Methods
7. Save as a convenient name

M. Create Sample set method

1. Select Browse Project, choose Project
2. Open samples tab
3. Set “Apply table preferences” to Sample set method
4. Insure drop down lists are Run and process and Continue on fault
5. Select row 1 – Function = Clear Calibration, enter method set name
6. Select row 2 – Function = Inject standards, enter method set name, number of injections = 3, Run Time = 4.00 minutes, injection volume =200
7. Save as a convenient name

N. Calibration Using an Existing Template (Project)

1. Open Run Samples and select Project and Chromatographic System (e.g. MBS HPLC 1)
2. Click on Load icon (Top left corner)
3. Select Load Using a Previously Created Set Method, click OK
4. Select Niclosamide Method , click open
5. Screen should be on the Samples tab with chosen template loaded
6. Press green button to start the standards injection sequence.
7. The Niclosamide template includes clearing previous calibrations and running a series of three 50 ppb standards
8. The injector should be returned to the LOAD position approximately 30 seconds after each injection.

O. Retrieving Results of Chromatograms

1. Select Browse Project.
2. Select the project to be viewed and click O.K.

3. Click on the results tab.
4. Double click on the chromatogram to be viewed.
5. If the chromatogram to be viewed is not available click on the update button.

II. Formulation of carrier

A. Preparation of acetic acid; 0.2 Molar:

1. Add about 200 mL distilled water (HPLC grade) to a 250 mL volumetric flask.
2. Dispense 2.9 mL glacial acetic acid into the volumetric flask.
3. Fill to the 250 mL mark with distilled water.
4. Label the volumetric flask.

B. Preparation of sodium acetate; 0.2 Molar:

1. Weigh 2.72 g sodium acetate (anhydrous), also known as acetic acid sodium salt,  $\text{CH}_3\text{CO}_2\text{Na}$ .
2. Add the sodium acetate to about 80 mL distilled water (HPLC grade) in a 100 mL volumetric flask.
3. Shake until the sodium acetate is dissolved, then fill the flask to the 100 mL mark with distilled water.
4. Label the volumetric flask.

C. Formulation of buffer; 0.01 Molar:

1. Add 41.0 mL of 0.2 Molar acetic acid and 9.0 mL of 0.2 Molar sodium acetate to a 1000 mL volumetric flask.
2. Fill to the 1000 mL mark with distilled water (HPLC grade).
3. Label the volumetric flask.

D. Formulation of carrier solution:

1. For Bayluscide analysis:
  - a. Add 220 mLs 0.01 Molar buffer solution to the carrier solvent reservoir.
  - b. Add 780 mLs methanol (HPLC grade) to the reservoir and mix
2. For TFM analysis:
  - a. Add 330 mLs 0.01 Molar buffer solution to the carrier solvent reservoir
  - b. Add 670 mLs methanol (HPLC grade) to the reservoir and mix
3. The sum of the volumes will be less than 1000 mL.
4. Larger volumes of carrier are made by adding buffer and methanol to the reservoir in the same proportion.

5. The solution should set over night with the cap open to allow degassing.
6. Label the container with % methanol, % buffer, date, and initials of formulator.

### III. Formulation of lampricide standards

#### A. Bayluscide standard

1. The number of niclosamide standards required for analysis is determined along with the proper concentration of each standard. The range of concentrations in the working standards includes and brackets the concentrations of niclosamide expected in the unknown water samples.
  - a. A single standard may be used for analysis of stream water samples. The response for concentrations of niclosamide in the normal working range is linear.
  - b. A series of three or more standards may be used as a check for precision of formulation of working standards.
2. For preparation of the working standards a sufficient volume (approximately 2000 mL) of stream water is filtered through Whatman 2V filter paper to remove solids that may cause interference during analyses (TOP:019.x).
3. A 0.50 mL sample of the 100 mg/L niclosamide field standard is dispensed into a 1000 mL volumetric flask which is filled to the volume mark with filtered stream water. An HPLC syringe may be used to dispense the standard. The precision measurement provided by the syringe will assure accuracy. Any syringe used to dispense field standard should be used ONLY to formulate standards and must be marked appropriately. The flask is stoppered and mixed. This dilution provides a 50 ug/L working standard.
4. The volumetric flask is labeled with date prepared, site from which the stream water was collected, concentration of niclosamide, and field standard code number.

#### B. TFM standard (50.0 ug/L)

1. Filter about 0.5 L stream water through Whatman 2V filter paper.
2. Dispense 5.0 mL of 1.0 mg/L field standard into a 100 mL volumetric flask. Fill to the 100 mL mark with filtered stream water.
3. Label the flask; include the code number of the TFM field standard used.

### IV. Quality assurance and documentation

- A. Each HPLC system is assigned a log book (Attachment). Every time the system is used an entry is made which documents use.
  1. The date, time, stream, and initials of the operator are recorded in the OPERATION LOG.
  2. The response of the instrument, standard concentration, retention time and pump pressure are also recorded in the OPERATION LOG for comparison with past and future applications.

3. Maintenance on any component of the system is recorded; any component substitution is noted on the COMPONENT LIST.
- B. All persons who operate the instrument receive training in analytical procedures. The trained operators sign the INITIALS page in each of the HPLC log books.
- C. Results of analyses are recorded on a Lampricide Analysis data form. These forms provide a permanent record of results.
- D. The method detection limit and limit of quantitation limit for the measurement of TFM concentrations are 2.4 ng/mL and 7.5 ng/mL, respectively (Hubert and Johnson, 2000).

#### **MAINTENANCE:**

The Waters 2489 detector may require replacement of the “keep alive” battery if an error screen is displayed during the systems check. This screen cannot be by-passed. Replace the 3 volt watch battery by:

**Note: to ensure safety, ensure that the system is unplugged before opening the detector.**

- I. Open the detector
  - A. Remove the four Phillips-head screws located on the bottom edge of the detector cover (two on each side).
  - B. Slide the metal outer cover off from the detector.
- II. Locate the battery
  - A. Locate on the mother board which sits upright on the right side of the detector.
  - B. The battery is located near the middle of the mother board and is held in place by a metal clip. The battery is silver, flat, and about the size of a quarter.
- III. Replace the battery
  - A. Any 3-volt type CR2032 replacement battery (Waters catalog number WAT080443) will work. Replacements may be purchased at any electronics store.
  - B. Lift the clip and place the new battery into position, noting the orientation of the old battery (a + sign should be visible when properly installed).
- IV. Close the unit
  - A. Slide the metal detector cover back in place.
  - B. Replace the four screws.
- V. Recalibrate the unit
  - A. Turn the detector on and wait for the unit to warm up (about five minutes).
  - B. Press shift/3 (calibrate).

- C. "Is the cuvette removed from its holder, and the flow cell prepared as required?" will be displayed
- D. Select the "ENTER" option.
- E. After the instrument completes internal checks and restorations "calibration successful" will be displayed.
- F. Select the "ENTER" option.
- G. The normal operating screen will appear.
- H. Perform a methanol flush to the system to complete the calibration.

Additional maintenance that can be conducted in the field is limited. Refer to the company manuals for guidance in troubleshooting or routine procedures.

**REFERENCES:**

Rheodyne 7725i Manual Injector Installation, Maintenance, and Troubleshooting Guide  
Waters 2489 Tunable Absorbance Detector Operator's Manuals  
Waters 515 HPLC Pump Operator's Guide  
Guide to Successful Operation of Your LC System  
Determination of TFM residues in sediment and water collected from the mouth and littoral zone of the Milakokia River (Schoolcraft and Mackinac counties, Michigan) during and following a lampricide application. 2000. Project Completion Report submitted to the Great Lakes Fisher Commission. (Unpublished) 19 p.

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This procedure has been reviewed and approved by the undersigned representatives of the U.S. Fish and Wildlife Service and Fisheries and Oceans Canada.

REVIEWED/APPROVED \_\_\_\_\_ DATE \_\_\_\_\_  
Field Supervisor (U.S.)

REVIEWED/APPROVED Luke Shivers DATE 05 MAR 2020  
Program Manager (Canada)