University of Kentucky, Biosystems and Agricultural Engineering Dept

HPLC STANDARD OPERATING PROCEDURE

Aminex 87-H and 87-P analytical columns

BAE
1/28/2015

BAE HPLC Standard Operating Procedure: 87H column
Last Updated: 12 December 2014

READ & UNDERSTAND THE ENTIRE SOP BEFORE BEGINNING

Note: An SOP cannot cover all the needed information for proper instrument understanding and operation, or cover all scenarios; nor can an SOP eliminate the necessity of awareness, attention to details and most importantly active thinking! Please refer to the instrument manuals for the needed background information, review literature, and always keep the most powerful instrument, the brain, turned on. When in doubt about something, ask!

HPLC System Information

1. Hardware: Dionex-Thermo Fisher Ultimate 3000 w/ auto-sampler(*HPLC #2*) , P-680 pump, TCC-100 Column Compartment, Variable Wavelength Detector (UV); Shodex R01 Refractive Index detector
2. Consumables: Analytical column Bio-Rad Aminex HPX-87H (order #125-0140) (ion exclusion); Guard column is a Bio-Rad Micro-Guard Cation-H cartridge (order #1250129); column typically operates at 50°C
3. The column type is specific to the analytes. The 87H column is a hydrogen-form column used for analysis of carbohydrates in solution with carboxylic acids, volatile fatty acids, short chain fatty acids, alcohols, ketones, and many neutral metabolic by-products. Most often used for organic acid analysis, this column is also useful for fermentation monitoring, biological fluid analysis, and acetylated amino sugar separations. See http://www.bio-rad.com/ for detailed information on other analytical column chemistry and purpose.

HPLC Operating Procedure

A. Mobile Phase (also known as working buffer, buffer, or solvent)

1. Flow rate is typically 0.4mL/min, constant flow rate (isocratic), typically a 20-70 minute run – run time will be a function of the elution time of the analytes being quantified.
2. Mobile phase is 5mM sulfuric acid for the 87H column. (Fisher #AC124645001)

B. Mobile Phase & Flush Water Preparation for the 87H Column

1. The mobile phase is defined by the column type – different columns may have a different mobile phase chemistry.
2. The volume of mobile phase/buffer needed will be calculated by Chromeleon after you've set up the run. See D.9 below. Confirm the amount needed, check the existing volume in the buffer rack on the HPLC, and make more if needed.
3. To make buffer, prepare a 500mM stock solution of sulfuric acid using the certificate of analysis to define the purity of the concentrated acid. Your calculations should yield a mixture of about 2.8mL of concentrated sulfuric acid into 97.2mL of double deionized water (DI water); note that the DI water unit has a 0.2 micron filter in-line.
4. Add 10mL of the 550mM stock solution to 990mL of (DI water) using a clean, DI water rinsed, glass 1L graduated cylinder to yield a 5mM final concentration.
5. Vacuum filter the buffer from the graduated cylinder through a 0.2µm x 47mm nylon filter (Pall Corporation, order #66602) to degas and clean buffer; use a glass funnel/support system (Pall

Corporation, order #4013 or #4012). Store filtered buffer into an appropriate container (1, 2 or 4L glass bottle with 33mm threaded top)

6. The purity of the buffer and flush water is critical – do not skip the filtration step. Poorly prepared, contaminated buffer can destroy a guard column, and/ or the analytical column.
7. The working buffer and flush water can be degassed if desired; however, the HPLC has a de-gassing pump so manual de-gassing is not needed.
8. Clearly LABEL all solution bottles with the chemical composition of the contents, the date prepared, and the name of the preparer.
9. Dionex recommends replacing aqueous solvents at least every two weeks. Do not 'top off' solvent bottles due to potential buildup of unwanted components. Do not let solvent bottles run dry! If a solvent bottle looks like it will be empty before the analysis is complete, stop the run between samples; see section D.1 for the requirements to change solvent bottles.

C. Standards & Standard Curves

NOTE: Standard curves are developed by the operator using calibration standards and are used by the HPLC software to quantify the concentration of analyte in the sample; the standards allow the operator to establish the elution time of analytes, and account for column drift or shifting elution times during analysis *(a normal occurrence)*. Calibration standards MUST be used with every HPLC analysis event.

1. Calibration standards (prepared samples with known concentrations used to calibrate the instrument) shall be prepared ahead of time for *each* analyte of interest. The calibration standards prepared for each analyte shall include not less than a two (2) low end concentrations, two (2) different mid-range concentrations, and a high end concentration for a total of five; this range of concentration values should bracket the expected range of concentrations in the sample. Typically, the concentrations should be from 70% of the lowest to 130% of the highest expected in the sample; note that blanks (zeroes) do not have a peak. A starting point to develop the appropriate standard concentrations would be to use literature values from multiple sources to establish the bracketing concentrations with multiple points around the typical concentrations reported. Ask the HPLC operator about the instrument detection limit(s) for your analytes. Note: the concentrations needed for the calibration samples will likely be different for each analyte.
2. The calibration is valid *only within* the concentration range of the standards used and not beyond it; thus samples that are outside the range of concentrations in the standards will produce unreliable results. A good understanding of the calibration process, linear regression, and statistics is of great value - see the "Theory of Calibration" in Chromeleon 6.80 User Manual, starting on page 109. The manual is found on the reference library CD (D:\library\manuals\software\CM_680_V30.pdf)
3. Combining multiple analytes into a single standard vial is acceptable, provided that there is good separation between peaks; if in doubt, make separate calibration samples. Ensure concentration calculations are correct – use the Certificate of Analysis (CoA) concentrations for the actual source bottle rather than the nominal concentration printed on the label. If the CoA concentration is not on the source bottle, it can usually be found on the supplier's website – use the Lot Number and catalog number printed on the label as search parameters.
4. All of the calibration standards shall be run at the beginning, and then unknown samples; provide a blank, a spiked sample, and a duplicate unknown sample for each run, or for every 15 to 17 unknown samples. The three extra samples are used for quality control (QC); these 18 to 20

samples represent a group of samples – the unknowns plus the quality control samples. The blank will be a sample that includes the background matrix, i.e., any reagents used in sample preparation, fresh microbial media, etc., but does not contain the analytes of interest [the blank provides a chromatogram of the background matrix]. The spiked sample should be prepared by splitting an existing unknown sample and then adding a known quantity of each analyte to the sample [the spike will show the background matrix effect on the analytes, if any exists]. The duplicate sample is a split unknown sample that can come from any unknown – it is often taken from the same sample that used in the spiking process [the duplicate will show instrument variability]. The quality control samples shall be evenly spaced among the unknown samples so that the QC check occurs throughout the grouping.

5. Provide at least one calibration verification sample per group or two per run. A calibration verification sample is a previously injected calibration standard that is injected again to check the accuracy and repeatability of the calibration and analytical results. The sample must be designated as a "validation sample" in the "type" column when setting up the sequence run – see D.1.8.
6. Run not less than one standard from each analyte group several times during the run in order to determine if the peak is shifting in time and to provide additional calibration points or calibration verification points. It is good practice to include more injections of the low end calibration standards to provide an average calibration point and some "weight" to balance the influence of the higher concentrations [see item 2 about the necessity of understanding the calibration (regression) process].
7. The FIRST calibration standard injection is a "waste" (the system uses this first injection as priming for the column so it never is as good as subsequent injections); use any of calibration standards because it will not be incorporated into the standard curve. The sequence could be S2, S1, S2, S3, S4, and so on, where the first S2 injection is the "waste" injection. The next three or more injections shall be standards loaded in order of increasing concentration.
8. One standard from each concentration grouping shall be run after the last sample at the end of the run. The total number of calibration standards, verification samples, QC samples,and unknown samples has to be considered to determine the needed buffer volume – Chromeleon will calculate the needed volume after the sequence has been set up.
9. For each group of unknown samples to be analyzed, randomly select not less than three but not more than 10 samples and analyze using the previously selected standards. Review the chromatograms to ensure that the correct analytes [all the peaks from the unknown samples are accounted for] and correct calibration standard concentrations have been chosen [all the unknown sample concentrations lie within the calibration standard concentrations]; adjust as needed before analyzing the remaining samples.
10. The best practice is to inject the smallest amount possible for both standards and unknown samples; it is suggested to start at 20 microliters and increase from there if there is not a sufficient response– use the injections described in C.9 to assess the response of a given concentration and injection volume – adjust as required.
11. The injection volume will determine how much sample/standard volume should be in the vial, e.g. a 100µl injection must have 600µl in the vial. Typically, the HPLC sample vials contain at least one mL. If the available sample volume is limited, there are inserts (250ul total volume) that fit inside the standard vials. If you're using an insert, adjust the injection volume on the sample setup page in the HPLC software, and change needle height. Failure to adjust the software setting for needle

height for vials with inserts will result in broken inserts and/or needles since the needle goes to the bottom of the vial when extracting the sample.

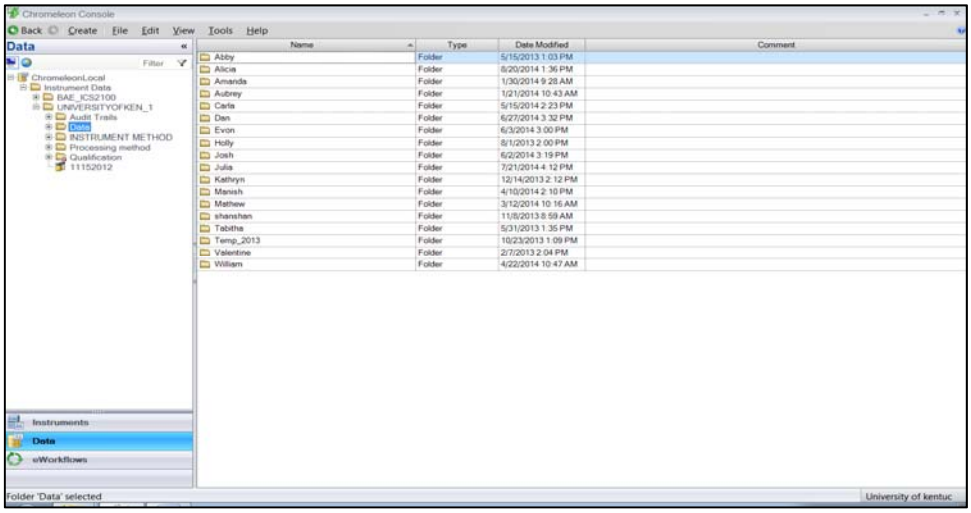
D. Instrument Preparation & Sample Analysis

NOTES:

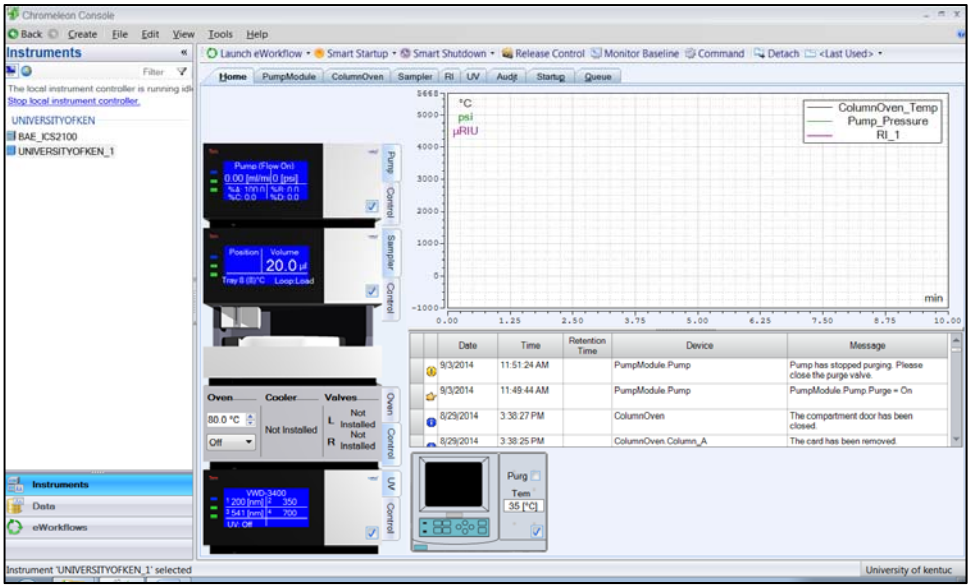
a. The following screen shots are in the appropriate order of operations – No Shortcuts! The mobile phase must be flowing before heating the column or warming the UV lamp.
b. The UV detector is not needed when analyzing only carbohydrates (sugars) on the 87H
c. Both the UV & RI detectors shall be used for fermentation analyses
d. Refer to the Chromeleon software manual for detailed information on creating new instrument methods and processing methods

I. Getting Started

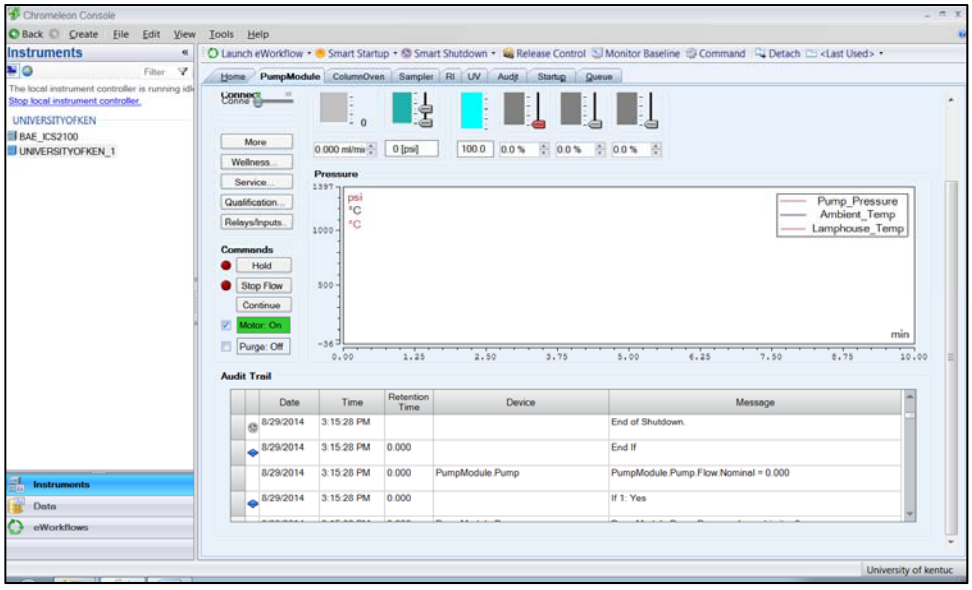
1. Open Chromeleon using the toolbar Icon. It will open to this screen:



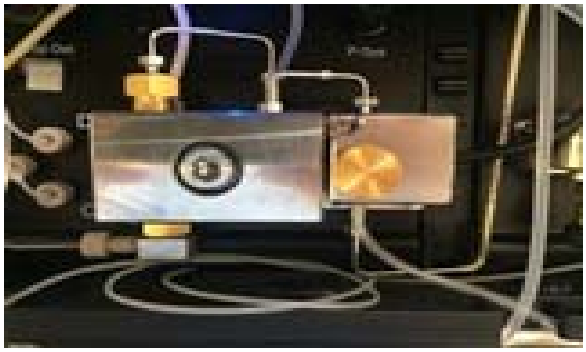
2. Select the instrument tab in the lower left corner, select UNIVERSITYOFKEN_1 in the left pane, and select the Home tab for general status.



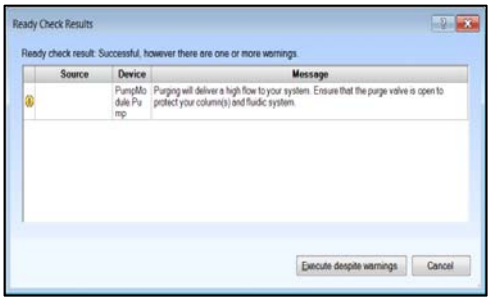
3. On the pump module tab, you can ensure that the pump is connected, set the flow rate, choose the mobile phase bottle, turn the pump on/off, and start a purge to set up a new solvent bottle.



a. To purge the lines of air after installing a new bottle of mobile phase (air in the lines is BAD), open the pump module door and turn the knurled brass knob two full turns (this bypasses the column and sends the flow directly to the waste container).

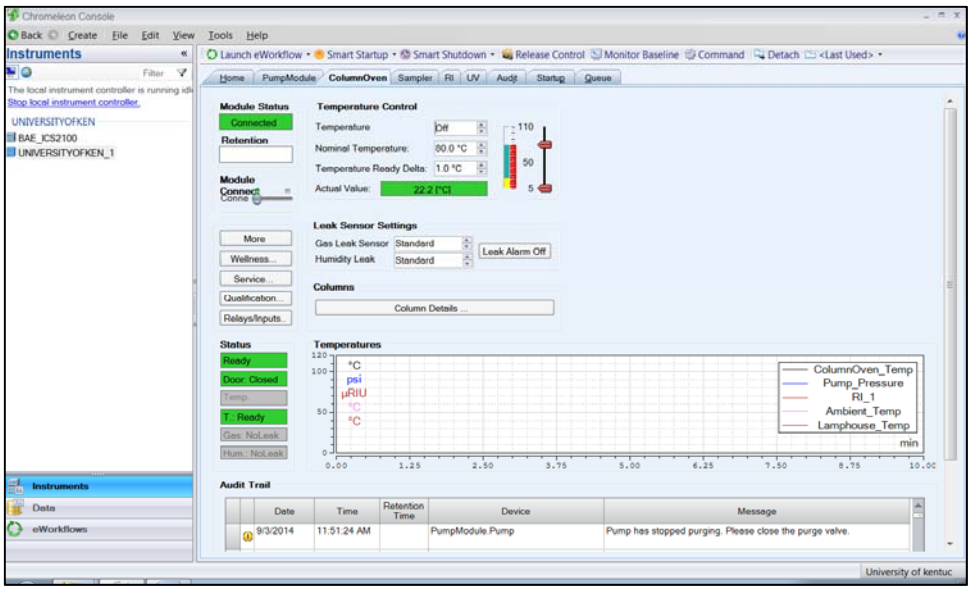


b. Then put a check in the purge box and this window will pop up. Start the purge – the purge process has a built in timer. If there is still visible air in the lines, purge a second time. Once the timer has elapsed, the purge button will go from green back to gray. Be sure to <u>CLOSE THE PURGE VALVE!</u>

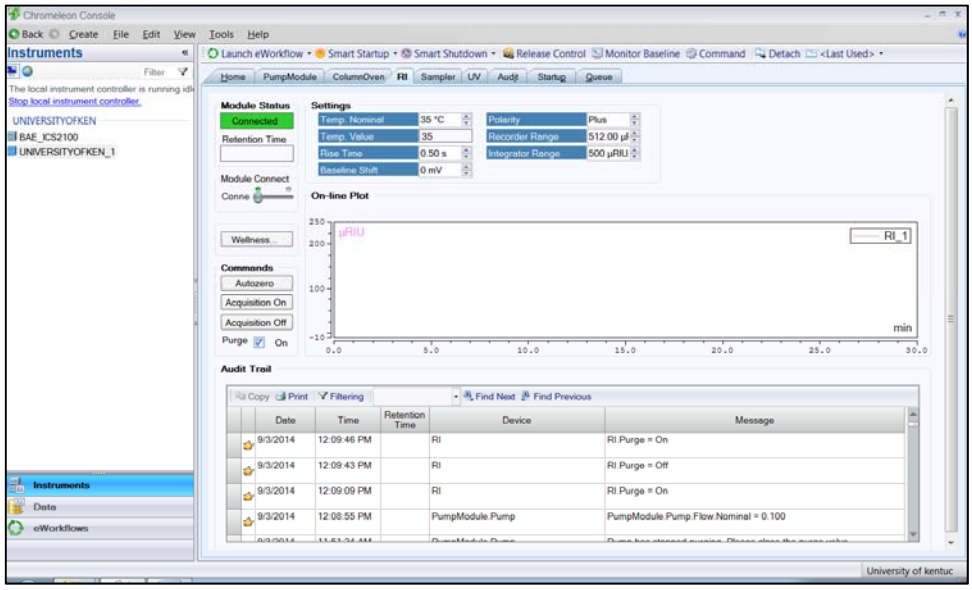


c. Now start initial flow through the column to prepare the system
   i. Check the motor on box – it will turn green
   ii. Set the flow rate at 0.1mL/min and let it flow for 30 minutes – the system pressure should be stable after 30 minutes – if not, continue flow at 0.1mL until pressure is stable.
   iii. Set the flow rate at 0.2mL/min for not less than 15 minutes (this ensures that the column is full before heating begins)

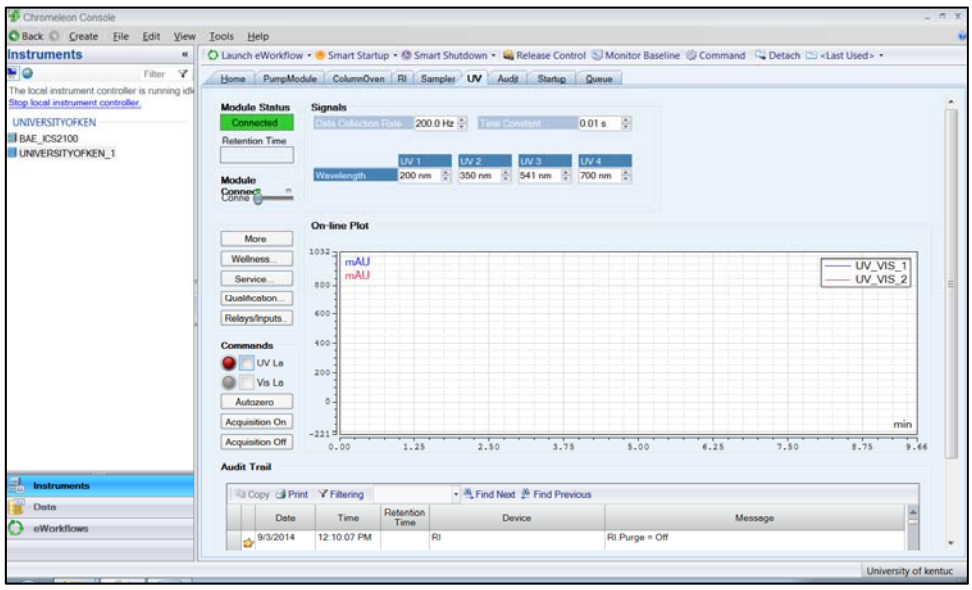
4. Go to the column tab, adjust the desired temperature set point and turn on "Temperature". After the column reaches the temperature set point, increase the flow in 0.100mL increments, wait <u>15-20 minutes, and repeat (increase flow rate, wait 15-20 min)</u> up to the final desired flow rate.



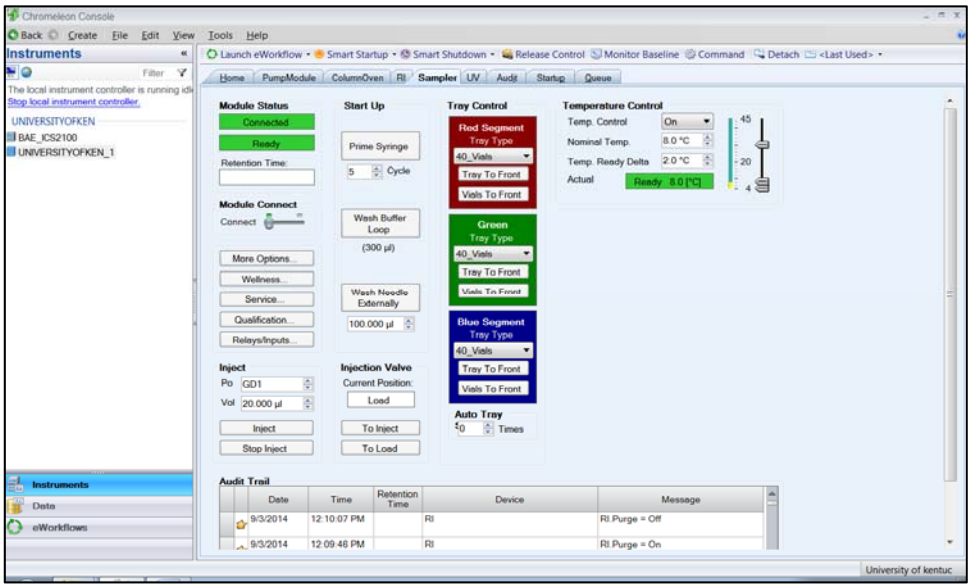
5. Go to the RI Tab and check the purge box; purge for not less than 5 minutes to remove air, different solvents, etc. <u>UNCHECK THE PURGE BOX BEFORE STARTING THE SAMPLE RUN SEQUENCE!</u>



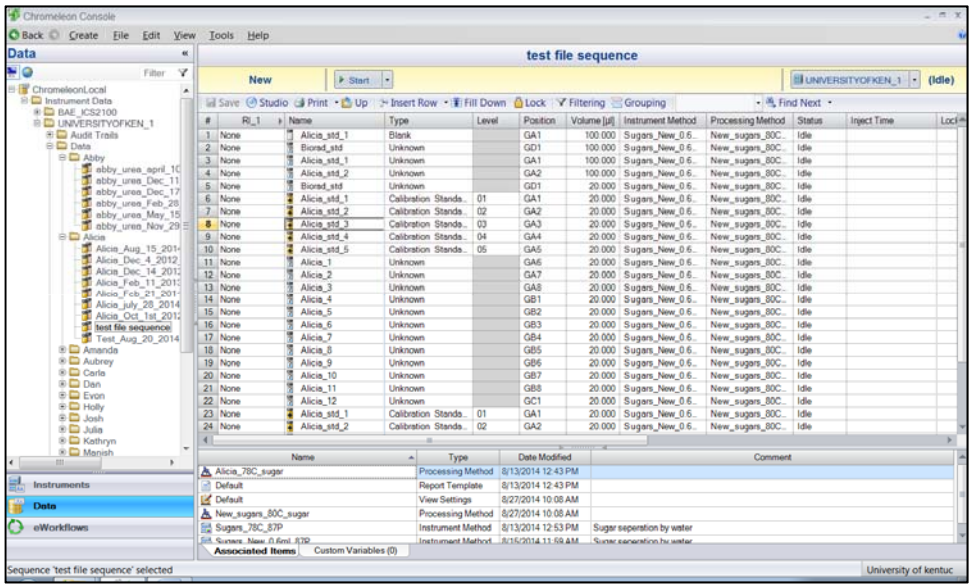
6. If using the UV detector, go to the UV tab and set the desired wavelength value in one of the 4 channels ,i.e., UV1 to UV4 (the channel used is specified in the instrument method used in a sequence). Turn on the UV lamp; it will take at least 30 minutes for the lamp to warm up –system will not proceed with the run until the lamp has reached operating temperature.



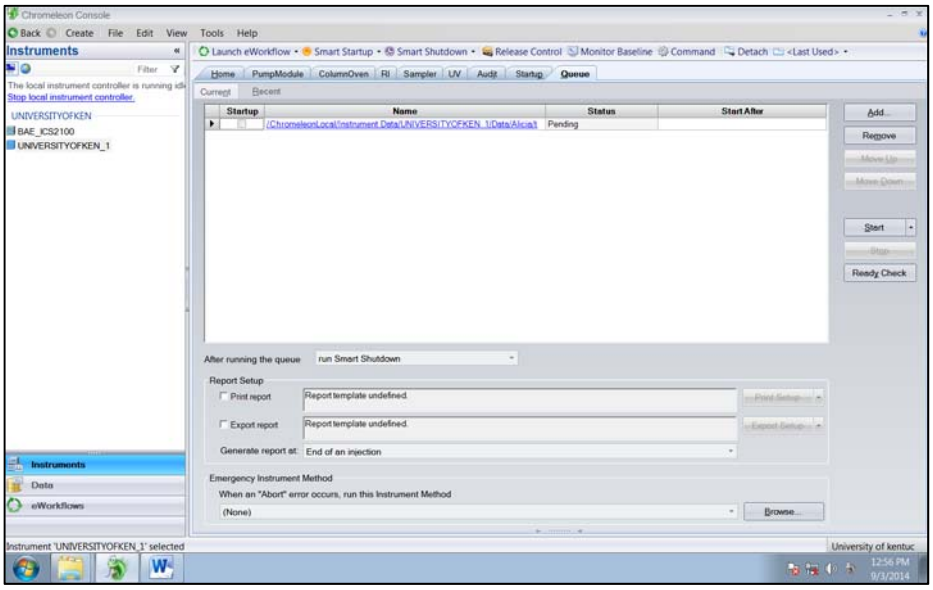
7. Go to the sample tab. Use the default tray control settings for each color (the colors represent sections of the tray). Set the tray temperature to the appropriate setting for your samples, e.g., 8°C is typical, however 4°may be appropriate for samples with microbes, or higher if needed. Use the "start up" and "inject" default values. The injection volume value (see C.9 above) will be overwritten by the value used in the sequence written for your samples.



8. Now that the instrument is ready, you can create a new sequence or open an existing sequence file from the data pane that has the same type of samples as yours and executing a save as "YYYYMMDD_file descriptor", e.g. "20140613_JohnDoe_Fermentation". For a new sequence, you Change the values in "name, type, and position" columns to match your samples. Use the appropriate injection volume (C.9 above). Use the existing instrument and processing methods unless you've created new ones. When using the UV, open the instrument method and confirm that the channel & wavelength are what you want. Select the "start" drop down menu and select "add sequence to the queue".



9. Go to the queue tab and select the "ready check" in the right pane. The ready check will calculate the needed mobile phase (a.k.a buffer, solvent) volume needed and display at the bottom of the window on the right side. Ensure that there is more buffer in the bottle than the calculated volume; if not see B.2 – B.9; once the buffer volume supply is confirmed then click the start button. The run will stop on its own once complete and use the "Smart shutdown" that is already programmed. Remove the samples and dispose of the vials in the proper location.



10. If there are errors during your run, consult with the HPLC operator, as the actions required depend on the nature of the problem. After the error is resolved, you can open the sequence, change the last sample from "interrupted" to "idle" and then save the sequence. Before restarting the run:
    a. Go to the console, sampler tab, select the wash buffer loop button. This will clean the flow path to ensure there is no cross sample contamination. Once the wash is complete (the wash buffer

loop button will change back to gray), restart the flow rate at 100uL and follow the flow rate incremental increase process discussed above in D.1.4. Once the flow rate is back to the desired value, restart the sequence.

II. Results Quality Control & Data Archival

NOTE: The area under the curve of each peak is used with the standard curve to generate a concentration value. Thus, any error in area delineation on the chromatogram and/or calculation directly and significantly impacts the results.

1. Standard Curves (calibration curves) shall be developed from the standards for each individual run – *do not re-use standard curves*. The default regression options in Chromeleon **MUST** be reviewed for each analysis event to ensure the chosen regression options make sense. The typical settings would be "linear with offset", "averaging all response values for each calibration level before curve fitting", and "no weighting". If any regression curve has an R-squared value of less than 0.999, investigate the problem and repeat the process to achieve an acceptable regression. See the Chromeleon 6.80 User Manual, starting on page 109. The manual is found on the reference library CD (D:\library\manuals\software\CM_680_V30.pdf) it provides excellent background information on calibration.
2. Chromeleon's automated tool can be used to establish baselines for each peak, identify the peak, and the drop lines for adjacent peaks. However, the software is only a starting point – the chromatogram for each sample shall be reviewed manually and corrected to ensure the proper placement of the lines defining the peak's areal boundary – the area definition is critical and requires intense focus.
3. The areal boundary lines (base lines and drop lines) may be defined manually using the HPLC software, the area values exported to excel, regressed, and concentrations calculated without using the Chromeleon algorithms.
4. The data (chromatogram file) from every sample analysis event **MUST** be archived in MS excel format on a storage device other than the computer operating the HPLC. The data shall be exported on a weekly basis or as needed (such as prior to a HPLC software update). The data in the archive **MUST** be maintained for a period of 10 years from the analysis date. The data shall be archived in primary folders by experimenter's name, then (years) and secondary folders (months). The MS Excel files shall include a primary identifier in the name, so the analysis type can be readily identified. The document properties dialog box is a convenient place to utilize keywords, etc., to facilitate a search function within the archive.

E. ROUTINE INSTRUMENT MAINTANENCE

Note: Refer to the equipment manuals for in-depth maintenance requirements including routine and periodic maintenance for the instruments and analytical columns. See the attached Bio-Rad Use & Care guide for both analytical and guard columns (BRUC).

I. HPLC Operation & Maintenance Logbook

1. A logbook shall be maintained by the operator for each HPLC system.
2. The logbook will be stored at each instrument's location and will be available for inspection
3. The logbook entries shall include the initials of the person making the entry, date, time, and notes regarding column (guard & analytical) maintenance, replacement, and performance, as well as

pertinent information about the samples being analyzed, along with system performance. All calls to Dionex and Bio-Rad technical support shall also be detailed in the log.

II. Guard & Analytical Columns

Note: The guard column maintenance and replacement frequency depends on the samples being analyzed and thus cannot be predicted. In general, replacement is warranted when standard samples illustrate a change in the measured data (resolution)

1. All column procedures (installation, start-up, & maintenance) shall be done per the BRUC.
2. A new guard column shall be installed when a new analytical column is installed.
3. The total system, analytical, and guard column pressures shall be determined at normal flow rates when newly installed and recorded in the logbook. Refer to the BRUC for the procedure to determine component pressures from system pressures.
4. Before any samples are analyzed, all newly installed columns shall be tested and the results compared to the factory supplied chromatogram to ensure proper column and system performance. Once the supplied and produced chromatograms match, samples analysis can begin. Both the supplier-produced and investigator-produced chromatographs should be dated and taped into the log book.
5. The HPLC pressure limit control (a operating software setting)shall be set so that a pressure increase of 15% greater than the pressure found in #3 (operating pressure) shall cause the pumps to stop.
6. The guard column shall be replaced if its pressure is greater than 150% of the pressure when new.
7. With a high pressure shutdown, a flush & regeneration cycle with the proper regeneration solvent shall be completed on the analytical column. See the BRUC table #2 for specifics and troubleshooting.
8. The System will need to have buffer run through it for not less than ½ hour once a week (if column is in place) when idle along with flushing pump line with dd water.
9. A gradual increase in column pressure is normal as the column ages and is proportional to the number of injections.
10. Lessons learned regarding column life & performance shall be incorporated to this SOP by the operator.

F. SOP MAINTANENCE

1. The HPLC operator shall review this standard operating procedure every 6 months and make all needed changes, and revise the date of update.
2. Dionex & Bio-Rad technical support solutions to specific problems shall be incorporated into the SOP.
3. Best practices learned by the HPLC operator for sample preparation, standards, standard ranges, etc., shall be communicated by the operator to the "owner" of the SOP for incorporation into the appropriate SOP.