| HBI Ion Labs a DCC business | Standard Operating Procedure<br>HPLC Operation Maintenance and Qualification | SOP Number D-807 | Revision 3 |
|---|---|---|---|
| | | Effective Date 06/02/25 | Page Page 1 of 17 |

| Written by/ Date | Reviewed by/ Date | Approved by/ Date |
|---|---|---|
| MSM 01/13/25 | AJS 01/13/25 | Pec 03/07/25 |
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## 1.0 Purpose

This procedure provides guidelines for general use, maintenance and qualification of HPLC systems.

## 2.0 Scope

This procedure applies to all HPLC systems used in the QC Laboratory at Ion Labs.

## 3.0 Responsibility

3.1 It is the responsibility of QC Laboratory analysts to follow the guidelines for general use of HPLC systems.

3.2 It is the responsibility of QC Laboratory Management to implement this procedure and to ensure that the procedure is being followed.

3.3 It is the responsibility of QC Laboratory Management, Analytical Development, and/or outside contractors to perform maintenance and qualification of HPLC instrumentation.

3.4 It is the responsibility of QC Laboratory Management and/or Analytical Development to keep this procedure current with the latest Ion Labs Practices.

## 4.0 Definitions

4.1 **QC** – Quality Control

4.2 **HPLC** – High Performance Liquid Chromatography

4.3 **PEEK** – Polyether Ether Keytone Polymer

4.4 **$KH_2PO_4$** – Potassium Phosphate Monobasic

4.5 **$H_3PO_4$** – Phosphoric Acid (~85%)

4.6 **ACN** – Acetonitrile

4.7 **IQ** – Installation Qualification

4.8 **OQ** – Operational Qualification

4.9 **PQ** – Performance Qualification

4.10 **UV** – Ultraviolet Wavelength Range Detector for HPLC

4.11 **PDA** – Photodiode Array Detector for HPLC

4.12 **RI** – Refractive Index Detector for HPLC

## 5.0 References

5.1 D-808, SOP, Use of OpenLab for HPLC and GC Data Acquisition and Reporting

5.2 D-603, SOP, Chemical Waste Disposal

## 6.0 Safety Precautions

6.1 Solvents used as mobile phases are toxic and flammable. The minimum required personal protective equipment includes safety glasses, gloves, and enclosed shoes. Ensure that solvent reservoirs and waste containers are gas-tight.

6.2 HPLC systems operate at high pressure. Compressed liquids may cause eye injury if a sudden leak occurs.

6.3 Electrical faults could cause electrocution, explosion or fire. If an electrical fault is suspected, disconnect power from the instrument and have it serviced by a qualified individual.

## 7.0 Waste Handling and Disposal

7.1 Waste handling and disposal procedures are outlined in SOP D-603.

## 8.0 General Guidelines

8.1 Solvent Compatibility

8.1.1 When changing from salt-containing (e.g. aqueous buffer) to high organic mobile phase or vice versa, it is important to flush the system with water in between. Otherwise, salt may precipitate in the system, causing blockage. Use a minimum of 30 mL flush solvent.

8.1.2 When changing between solvents that are not miscible (e.g. hexane to water), it is important to flush the system with an intermediate solvent that is miscible with both solvents. Isopropanol is a good intermediate solvent since it is miscible with all common solvents used for HPLC. Use a minimum of 30 mL flush solvent.

8.2 Sample Filtration and/or Centrifugation

8.2.1 Samples to be injected into the HPLC system must be free of all particulates.

8.2.2 Filtration is the preferred method for removal of particulates. For columns with particle diameter of 3 µm or larger, a filter with 0.45 µm membrane is adequate. For columns with particle diameter less than 3 µm, use a filter with 0.2 µm membrane.

8.2.3 Alternatively, centrifugation may be used to remove particulates provided that the resulting solution is clear.

8.3 Extra column Volume

8.3.1 Extra column volume is the mobile phase volume that a solute experiences outside of the column. Unnecessary extra column volume can lead to degradation of peak shape.

8.3.2 Extra column volume can be minimized by paying particular attention to the fittings and tubing used between the autosampler, column, and detector.

8.3.2.1 The internal diameter of HPLC tubing for most applications should be no more than 0.010" internal diameter. Tubing is color coded to indicate internal diameter: natural = 0.0025", red = 0.005", yellow = 0.007", blue = 0.010". Smaller internal diameter tubing minimizes extra column volume, but clogs easier.

8.3.2.2 HPLC fittings must be properly connected to minimize extra column volume.

8.3.2.2.1 Stainless steel fittings are often used for (semi)permanent connections. Stainless steel fittings consist of a nut and a ferrule, which come in numerous

shapes and sizes. It is best to use fittings that are designed for the specific manufacturer's equipment. Additionally, stainless steel fittings must be permanently attached to the tubing (swaged). To do this, place the nut and ferrule (in that order) onto the tubing, insert the end of the tubing into the port until it bottoms, finger tighten the nut, and then tighten with a wrench an additional ¾ turn.

8.3.2.2.2 Polymeric fittings (most commonly PEEK) are more universal and do not have the shape and size limitations of stainless steel fittings. They do not permanently attach to tubing; and therefore, are often used for attaching HPLC columns. To properly attach a polymeric fitting: place the fitting on the tubing, insert the tubing into the port until it bottoms, and tighten the fitting while keeping the tubing completely inserted in the port.

8.4 Mobile Phase pH

8.4.1 HPLC columns are typically stable within the pH range of 2.0 – 8.0. Consult the column care guide for your specific column if you intend to use a mobile phase outside of this pH range to avoid permanent damage to the column packing.

8.5 Column pressure

8.5.1 Many HPLC columns can be irreversibly damaged by pressures greater than 3500 psi (~250 bar). Consult the column care guide for your specific column if you intend to use pressure greater than 3500 psi.

9.7 Mobile phase channels that will not be used for extended periods of time (> 1 week) should be purged and stored in isopropanol.

9.8 Download the method to the instrument, and begin equilibrating the system under initial conditions. After a stable baseline is obtained, the run may be started.

9.9 Refer to SOP D-808 Use of OpenLab for HPLC and GC Data Acquisition and Reporting, for instructions detailing how to acquire, process, and report using OpenLab.

9.10 After the run has completed, prepare the column for storage:

9.10.1 Reverse Phase Methods (e.g. C18, C8, C5, Phenyl)

9.10.1.1 If a mobile phase containing salts was used:

9.10.1.1.1 Open the purge valve, and purge each channel containing salts with at least 30 mL of water / acetonitrile (90 / 10).

9.10.1.1.2 Close the purge valve, and allow the system to pump each channel containing salts at the flow rate specified in the method for at least 10 minutes.

9.10.1.2 Open the purge valve, and purge each channel that was used with at least 30 mL of acetonitrile / water (50/50).

9.10.1.3 Close the purge valve, and allow the system to pump at the flow rate specified in the method for at least 10 minutes.

9.10.1.4 Discontinue flow, remove the column, and replace the column end-caps.

9.10.2 Normal Phase Methods (e.g. Un-bonded Silica Gel, Alumina, Amino or Cyano)

9.10.2.1 The column may be stored in mobile phase. Discontinue flow, remove the column, and replace the column end-caps.

## 10.0 Preventative Maintenance

10.1 The following is a general list of items typically included in regular preventative maintenance of HPLC systems. Consult the specific instrument manual for specific instructions on how to perform each maintenance step.

10.2 Inspect the system, and use a damp paper towel to clean any dust, salt, or sample residue that may be present.

10.3 Pump and degasser

10.3.1 Connect a flow restrictor in place of the column.

10.3.2 Purge all channels with DI water, and pump DI water at 1 mL/min for at least 15 minutes.

10.3.3 Clean or replace solvent inlet filters when excessive air bubbles are observed in the mobile phase tubing or when filters appear dirty. Glass filters may be soaked in 35% nitric acid for one hour and flushed thoroughly with water to clean them.

10.3.4 Replace purge valve frit as necessary.

10.3.5 If the pump has a seal wash feature, replace wash seals/gaskets annually.

10.3.6 Clean or replace the outlet check valve annually.

10.3.7 Clean or replace the inlet check valve annually.

10.3.8 Inspect and clean pistons annually. Replace as necessary.

10.3.9 Replace pump piston seals annually.

10.3.10 Perform seal wear-in procedure if required when seals are replaced.

10.3.11 Purge all channels with isopropanol, and pump at 1 mL/min for at least 1 hour. It is recommended to pump isopropanol through the system at a reduced flow rate overnight.

10.4 Autosampler

10.4.1 Inspect and replace needle as necessary.

10.4.2 Replace needle seat annually.

10.4.3 Replace rotor seal annually.

10.4.4 Replace metering seal annually.

10.4.5 Inspect and replace the finger caps (vial gripper) as necessary.

10.4.6 Perform seal wear-in procedure if required when seals are replaced.

10.5 UV and PDA Detectors

10.5.1 Replace the lamp(s) as necessary.

10.5.2 Clean or replace the flow cell as necessary.

10.6 RI Detectors

10.6.1 RI detectors do not have any user serviceable parts.

## 11.0 Performance Qualification

11.1 The following tests are recommended for annual performance qualification (PQ) of HPLC systems. Installation qualification (IQ) and operational qualification (OQ) are typically performed by the instrument vendor.

11.2 Supplies needed

11.2.1 Acetone

11.2.2 KH2PO4

11.2.3 H3PO4

11.2.4 ACN

11.2.5 Caffeine reference standard

11.2.6 Glycerin reference standard

11.2.7 Calibrated temperature sensor

11.2.8 Calibrated timer or stopwatch

11.2.9 Analytical balance capable of weighing to the nearest 0.1mg

11.2.10 Acclaim 120 C18 5µm column, 4.6 x 250mm

11.2.11 Gemini C6-Phenyl 5 µm column, 4.6 x 250mm (for RI detector calibration)

11.2.12 Flow restrictor (PEEK tubing, 0.007 mm id, 5 m length)

11.2.13 25-mL graduated cylinder

11.2.14 50-mL graduated cylinder

11.2.15 Seals, pistons, frits as necessary.

11.3 Pump accuracy

11.3.1 Attached the flow restrictor in place of the column.

11.3.2 Purge the system with degassed DI water.

11.3.3 Start the flow at 1 mL/min.

11.3.4 Place a clean, dry, pre-weighed 25 mL graduated cylinder near the outlet of the flow restrictor.

11.3.5 Simultaneously start a calibrated timer and place the outlet of the flow restrictor into the graduated cylinder.

11.3.6 After exactly 15 min, remove the outlet from the graduated cylinder.

11.3.7 Record the weight of the cylinder and water.

11.3.8 Record the temperature of the water contained in the cylinder.

11.3.9 Using the table below, calculate the volume of water collected and the flow rate.

11.3.10 Repeat the experiment at 3 mL/min.

11.3.11 Acceptance criteria: nominal flow rate ± 2%.

**Table: Density of Water from 15°C – 30°C in g/mL**

| | 0 | 0.1 | 0.2 | 0.3 | 0.4 | 0.5 | 0.6 | 0.7 | 0.8 | 0.9 |
|---|---|---|---|---|---|---|---|---|---|---|
| 15 | 0.9991 | 0.9991 | 0.9991 | 0.9991 | 0.9990 | 0.9990 | 0.9990 | 0.9990 | 0.9990 | 0.9990 |
| 16 | 0.9989 | 0.9989 | 0.9989 | 0.9989 | 0.9989 | 0.9989 | 0.9988 | 0.9988 | 0.9988 | 0.9988 |
| 17 | 0.9988 | 0.9988 | 0.9987 | 0.9987 | 0.9987 | 0.9987 | 0.9987 | 0.9987 | 0.9986 | 0.9986 |
| 18 | 0.9986 | 0.9986 | 0.9986 | 0.9985 | 0.9985 | 0.9985 | 0.9985 | 0.9985 | 0.9984 | 0.9984 |
| 19 | 0.9984 | 0.9984 | 0.9984 | 0.9983 | 0.9983 | 0.9983 | 0.9983 | 0.9983 | 0.9982 | 0.9982 |
| 20 | 0.9982 | 0.9982 | 0.9982 | 0.9981 | 0.9981 | 0.9981 | 0.9981 | 0.9981 | 0.9980 | 0.9980 |
| 21 | 0.9980 | 0.9980 | 0.9979 | 0.9979 | 0.9979 | 0.9979 | 0.9979 | 0.9978 | 0.9978 | 0.9978 |
| 22 | 0.9978 | 0.9977 | 0.9977 | 0.9977 | 0.9977 | 0.9977 | 0.9976 | 0.9976 | 0.9976 | 0.9976 |
| 23 | 0.9975 | 0.9975 | 0.9975 | 0.9975 | 0.9974 | 0.9974 | 0.9974 | 0.9974 | 0.9973 | 0.9973 |
| 24 | 0.9973 | 0.9973 | 0.9972 | 0.9972 | 0.9972 | 0.9972 | 0.9971 | 0.9971 | 0.9971 | 0.9971 |
| 25 | 0.9970 | 0.9970 | 0.9970 | 0.9970 | 0.9969 | 0.9969 | 0.9969 | 0.9969 | 0.9968 | 0.9968 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 26 | 0.9968 | 0.9968 | 0.9967 | 0.9967 | 0.9967 | 0.9966 | 0.9966 | 0.9966 | 0.9966 | 0.9965 |
| 27 | 0.9965 | 0.9965 | 0.9965 | 0.9964 | 0.9964 | 0.9964 | 0.9963 | 0.9963 | 0.9963 | 0.9963 |
| 28 | 0.9962 | 0.9962 | 0.9962 | 0.9961 | 0.9961 | 0.9961 | 0.9961 | 0.9960 | 0.9960 | 0.9960 |
| 29 | 0.9959 | 0.9959 | 0.9959 | 0.9959 | 0.9958 | 0.9958 | 0.9958 | 0.9957 | 0.9957 | 0.9957 |
| 30 | 0.9956 | 0.9956 | 0.9956 | 0.9956 | 0.9955 | 0.9955 | 0.9955 | 0.9954 | 0.9954 | 0.9954 |

11.4 Column Oven Temperature

11.4.1 Set the temperature of the column oven to 35 °C.

11.4.2 Place a calibrated temperature-sensing device in the column oven with the probe near the instruments oven thermocouple.

11.4.3 Record the temperature reading after it has stabilized.

11.4.4 Repeat the experiment at 50°C.

11.4.5 Acceptance criteria: nominal temperature ± 2°C.

11.5 Detector Wavelength Accuracy (UV and PDA detectors)

11.5.1 Turn the detector lamps on and allow them to warm up for at least 30 min.

11.5.2 Connect the restrictor tubing in place of the column.

11.5.3 Prime the pump with DI water, and set the flow rate to 2.0 mL/min.

11.5.4 Set data acquisition for a 1 minute run with a sampling period of about 400 milliseconds or 2.5 points per second.

11.5.5 Set the detector to record spectral data over the range 200 – 300 nm with step of 1 nm and slit width of 1 nm. If the slit width cannot be set to 1 nm, use the lowest possible setting. Slit width may not be controllable on some detectors.

11.5.6 Inject 10 µL of a 25 µg/mL caffeine solution and record the wavelength of maximum absorbance.

11.5.7 Acceptance criteria: 205 ± 2 nm and 273 ± 2 nm

11.6 Detector Noise and Drift (UV and PDA detectors)

11.6.1 If not already on, turn the detector lamps on and allow them to warm up for at least 30 min.

11.6.2 Connect the restrictor tubing in place of the column.

11.6.3 Prime the pump with DI water, and set the flow rate to 1.0 mL/min.

11.6.4 Set data acquisition for a 10 minute run with a sampling period of about 400 milliseconds or 2.5 points per second.

11.6.5 Set the wavelength to 273 nm with slit width of 3 nm. If the slit width cannot be set to 3 nm, use the closest possible setting. Slit width may not be controllable on some detectors.

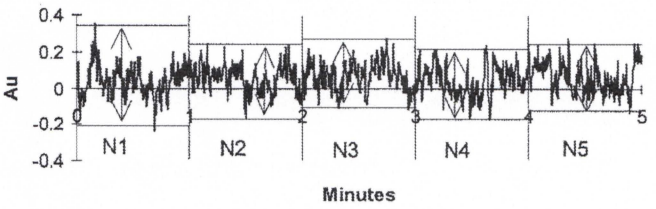
11.6.6 Perform a null injection (set Injection Source to No Injection in the sequence table) and print the resulting chromatogram.

11.6.7 Calculate the system noise using 5 consecutive minutes of data.

11.6.8 Measure the noise level for each one minute interval as shown below.

11.6.9 Calculate the average noise level for the five one minute intervals.

11.6.10 Noise calculation example:



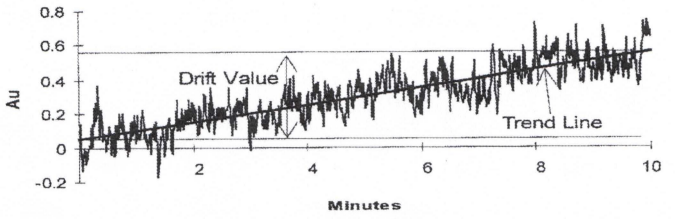

11.6.11 Noise acceptance criteria: NMT 200 (µV or µAU).

11.6.12 Calculate the system drift using all 10 minute of data.

11.6.13 Multiply the drift value by 6 to obtain the drift per hour.

11.6.14 Drift acceptance criteria: NMT 3.00 (mV/hr or mAU/hr).

11.6.15 Drift calculation example:




11.1 Detector Noise and Drift (RI detectors)

11.1.1 Connect a Gemini C6-Phenyl 5 µm, 4.6 x 250mm column.

11.1.2 Prime the pump with DI water, and set the flow rate to 1.0 mL/min.

11.1.3 Set data acquisition for a 10 minute run.

11.1.4 Set the column temperature to 30°C.

11.1.5 Set the detector peakwidth to a 4 sec response time.

11.1.6 Set the RI optical unit temperature to 35°C.

11.1.7 Set Automatic Zero Before Analysis to ON.

11.1.8 Set Automatic Recycling After Analysis to OFF.

11.1.9 Start flow of DI water, and wait for the baseline to stabilize.

11.1.10 Purge the reference cell for at least 10 minutes.

11.1.11 Turn purge reference cell to Off, and wait for the baseline to stabilize. It is recommended to allow the system to equilibrate overnight prior to evaluating noise and drift. To do this, continue the flow of DI water at 1.0 mL/min and switch the recycling valve to on. Make sure that the recycle tubing coming from the detector is in the mobile phase container. After equilibration, turn the recycling valve off.

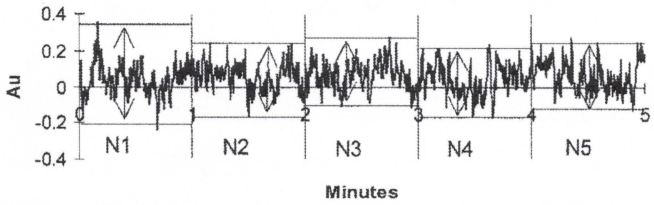
11.1.12 Perform a null injection (set Injection Source to No Injection in the sequence table) and print the resulting chromatogram.

11.1.13 Calculate the system noise using 5 consecutive minutes of data.

11.1.14 Measure the noise level for each one minute interval as shown below.

11.1.15 Calculate the average noise level for the five one minute intervals.

11.1.16 Noise calculation example:



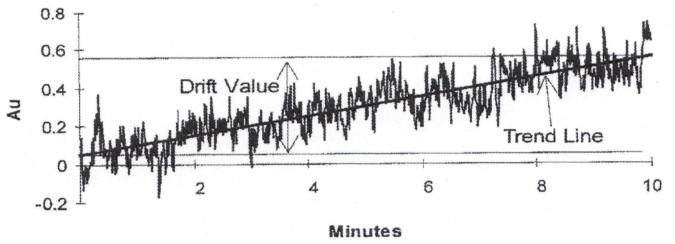

11.1.17 Noise acceptance criteria: NMT 20 nRIU.

11.1.18 Calculate the system drift using all 10 minute of data.

11.1.19 Multiply the drift value by 6 to obtain the drift per hour.

11.1.20 Drift acceptance criteria: NMT 400 nRIU/hr.

11.1.21 Drift calculation example:




11.2 Injector Precision and Carryover (UV and PDA detectors)

11.2.1 If not already on, turn the detector lamps on and allow them to warm up for at least 30 min.

11.2.2 Connect an Acclaim 120 C18 5µm column, 4.6 x 250 mm.

11.2.3 Prepare the Mobile Phase

11.2.3.1 Transfer 1.36g of KH2PO4 to a 1000-mL mobile phase bottle.

11.2.3.2 Add 750 mL H2O, and stir to dissolve.

11.2.3.3 Adjust to pH 3.1 – 3.4 using H3PO4.

11.2.3.4 Add 250 mL of ACN.

11.2.3.5 Filter using 0.45 µm nylon membrane.

11.2.4 Prime the pump with Mobile Phase, and set the flow rate to 1.0 mL/min.

11.2.5 Set the detector wavelength to 273 nm with a slit width of 3 nm. If the slit width cannot be set to 3 nm, use the closest possible setting. Slit width may not be controllable on some detectors.

11.2.6 Set the injection volume to 10 µL.

11.2.7 Set the column temperature to 35°C.

11.2.8 Set data acquisition for a 7 minute run with a sampling period of about 200 milliseconds or 5 points per second.

11.2.9 Perform 10 replicate injections of of a 25 µg/mL caffeine solution followed by a single blank (DI water) injection.

11.2.10 Calculate the %RSD of six consecutive injections.

11.2.11 Calculate the percent carryover as the peak area of the blank injection divided by the peak area of the immediately preceding injection of caffeine solution multiplied by 100.

11.2.12 Precision acceptance criteria: NMT 1.0 % RSD.

11.2.13 Carryover acceptance criteria: NMT 0.1 %.

11.3 Detector Linearity (UV and PDA detectors)

11.3.1 If multiple flow cells are available for the detector, install the flow cell with lowest sensitivity.

11.3.2 If not already on, turn the detector lamps on and allow them to warm up for at least 30 min.

11.3.3 Connect the restrictor tubing in place of the column.

11.3.4 Prime the pump with DI water, and set the flow rate to 2.0 mL/min.

11.3.5 Set the detector wavelength to 273 nm with a slit width of 3 nm. If the slit width cannot be set to 3 nm, use the closest possible setting. Slit width may not be controllable on some detectors.

11.3.6 Set data acquisition for a 1 minute run with a sampling period of 200 milliseconds or 5 points per second.

11.3.7 Perform a blank injection followed by injections of a 25 µg/mL caffeine solution at six injection volumes varying from 5% to 100% of the installed sample loop.

11.3.8 Report the peak areas and calculate the coefficient of determination (r2).

11.3.9 Linearity acceptance criteria: NLT 0.999 for $r^2$.

11.4 Refractive Index Sensitivity and Linearity

11.4.1 Prepare a 1.0 mg/mL glycerin solution by dissolving 50 mg of glycerin reference standard in 50 mL of DI water.

11.4.2 Prepare a 0.1 mg/mL glycerin solution by diluting 5.0 mL of 1.0 mg/mL glycerin solution to 50 mL using DI water.

11.4.3 Connect a Gemini C6-Phenyl 5 µm, 4.6 x 250mm column.

11.4.4 Prime the pump with DI water, and set the flow rate to 1.0 mL/min.

11.4.5 Set data acquisition for a 10 minute run.

11.4.6 Set the column temperature to 30°C.

11.4.7 Set the detector peakwidth to a 4 sec response time.

11.4.8 Set the RI optical unit temperature to 35°C.

11.4.9 Set Automatic Zero Before Analysis to ON.

11.4.10 Set Automatic Recycling After Analysis to OFF.

11.4.11 Start flow of DI water, and wait for the baseline to stabilize.

11.4.12 Purge the reference cell for at least 10 minutes.

11.4.13 Turn purge reference cell to Off, and wait for the baseline to stabilize. It is recommended to allow the system to equilibrate overnight prior to evaluating noise and drift. To do this, continue the flow of DI water at 1.0 mL/min and switch the recycling valve to on. Make sure that the recycle tubing coming from the detector is in the mobile phase container. After equilibration, turn the recycling valve off.

11.4.14 For sensitivity: perform a single injection of the 0.1 mg/mL glycerin standard. Calculate the signal-to-noise ratio for the glycerin peak using at least one minute of baseline for measurement of noise.

11.4.15 For linearity: perform six injections of 1.0 mg/mL glycerin standard with injection volumes ranging from 5% - 100% of the sample loop volume. Plot peak area versus injection volume, and calculate $R^2$ for a linear regression.

11.4.16 Sensitivity Acceptance Criteria: S/N is NLT 10.

11.4.17 Linearity Acceptance Criteria: $R^2$ if NLT 0.995.

11.5 Gradient Accuracy

11.5.1 Fill two 1-L mobile phase bottles with DI water. Sonicate for 15 minutes.

11.5.2 Fill two 1-L mobile phase bottles with 0.1% acetone in DI water. Sonicate for 15 minutes.

11.5.3 Set mobile phase channels A and C with DI water.

11.5.4 Set mobile phase channels B and D with 0.1% acetone in DI water.

11.5.5 Purge A/B/C/D (25/25/25/25) at 5 mL/min for 45 min.

11.5.6 Set the UV detector wavelength to 265 nm.

11.5.7 Set the solvent program as follows:

| Time (min) | Flow Rate (mL/min) | % A | % B | % C | % D |
|---|---|---|---|---|---|
| 0.00 | 4 | 100 | 0 | 0 | 0 |
| 2.99 | 4 | 100 | 0 | 0 | 0 |
| 3.00 | 4 | 75 | 25 | 0 | 0 |
| 5.99 | 4 | 75 | 25 | 0 | 0 |
| 6.00 | 4 | 50 | 50 | 0 | 0 |
| 8.99 | 4 | 50 | 50 | 0 | 0 |
| 9.00 | 4 | 0 | 100 | 0 | 0 |
| 11.99 | 4 | 0 | 100 | 0 | 0 |
| 12.00 | 4 | 0 | 50 | 50 | 0 |
| 14.99 | 4 | 0 | 50 | 50 | 0 |
| 15.00 | 4 | 0 | 25 | 75 | 0 |
| 17.99 | 4 | 0 | 25 | 75 | 0 |
| 18.00 | 4 | 0 | 0 | 100 | 0 |
| 20.99 | 4 | 0 | 0 | 100 | 0 |
| 21.00 | 4 | 0 | 0 | 75 | 25 |
| 23.99 | 4 | 0 | 0 | 75 | 25 |
| 24.00 | 4 | 0 | 0 | 50 | 50 |
| 26.99 | 4 | 0 | 0 | 50 | 50 |
| 27.00 | 4 | 0 | 0 | 0 | 100 |
| 30.00 | 4 | 0 | 0 | 0 | 100 |

11.5.8 Begin flow at 100% A and 4 mL/min. Wait for the absorbance reading to stabilize (at least 10 min).

11.5.9 Begin the run with a null injection (set Injection Source to No Injection in the sequence table).

11.5.10 Print the chromatogram and measure in millimeters the height from the baseline of each step.

11.5.11 Calculate the mixing accuracy by dividing the height at the 25% and 50% steps by the height at the 100% step and multiplying by 100.

11.5.12 Mixing accuracy acceptance criteria: nominal value ± 2.0%.

## 12.0 Revision History

| Revision | Date | Description of Changes | CCR # | By |
|---|---|---|---|---|
| 0 | 02/10/20 | New | N/A | S. Sassman |
| 1 | 09/07/22 | Use a column instead of flow restrictor for precision and carryover PQ test, fix the table for gradient accuracy, calculate flow rate by weight instead of volume, include options for slit width setting, add performance qualification evaluation for refractive index detectors. | CC-22-0363 | S. Sassman |
| 2 | 12/20/22 | Added reference and instructions for HPLC/ GC Checklist. | CC-22-0476 | J. Sassman |
| 3 | 01/13/25 | Remove reference and instructions for HPLC/GC Checklist | CC-24-0522 | Melisa Maples |