Guideline for HPLC Sample Analysis

High-performance liquid chromatography (HPLC), formerly referred to as high-pressure liquid chromatography, is a technique in analytical chemistry used to separate, identify, and quantify component in a mixture. The photo diode array (PDA), also known as the diode array detector (DAD) can measure the entire wavelength range (200-800 nm) in real time, which provide UV-Vis spectra of analytes.

Waters alliance e2695 HPLC system

Developed for integrated HPLC-PDA for qualitative applications only for UV-vis active compounds.

Specification:

1.Flow: 0.1-10.0 ml/min
2.PDA scan range: 200-800nm
3.Resolution: 1.2 nm.
4.Accuracy: 1 nm
5.Acquisition speed: 1-80 Scan/second 6.Sensitivity: 1µg/ml

HPLC/PDA SAMPLE SUBMISSION REQUIREMENTS

1. Sample Quantity & Quality

- Submit **5–10 mg** of clean, properly prepared **solid or lyophilized sample**.
- Samples must be free from **salts, detergents, polymers, and other MS-incompatible contaminants**.

2. Buffer & Solvent Compatibility

- Use **HPLC-grade solvents and reagents** for sample preparation, wherever applicable.

3. Sample Identification

- Clearly label every sample with a unique **Sample ID / Code**.
- The **same Sample ID** must appear on both the **submission form and sample container**.

4. Sample Container

- Submit samples in **properly sealed, clean, and compatible vials/tubes**.
- Prevent leakage, and contamination during transport.

5. User Responsibility

- Users are responsible for providing **accurate and complete sample information**.
- Inadequately labelled, contaminated, or improperly prepared samples may be **returned for re-preparation**.

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Spiked Standard Method (Qualitative Analysis)

1. Purpose

The spiked standard method is used for the **qualitative identification of a suspected compound in a sample** by comparing its HPLC retention time (RT) with that of an authentic reference standard.

2. Principle

The sample is analyzed by HPLC under defined chromatographic conditions. A solution of the suspected reference standard is analyzed separately and then added (spiked) to the sample. A compound may be considered tentatively identified when the sample peak and standard peak have the **same or closely matching retention time** and the corresponding peak increases in intensity after spiking, without the appearance of an unexpected additional peak.

3. Sample and Standard Requirements

- Submit 2-5 mg clearly labelled sample.
- The sample should be completely soluble in the mobile phase or compatible RP HPLC solvents.
- Provide an authentic reference standard (2-3 mg) of the targeted compound, preferably with documented purity.

4. Interpretation of Results

Observation	Interpretation
Sample has no peak at the standard RT	Compound is not detected under the applied conditions
Sample peak occurs at similar RT to standard	Compound may be present; further confirmation is recommended
Peak at the same RT becomes substantially larger after spiking	Supports the presence of the suspected compound
New separate peak appears after spiking	Sample peak may represent a different compound
RT differs significantly	Identification is not supported
Multiple/overlapping peaks occur	HPLC conditions may require optimization

5. Remarks

Retention time alone should not be considered definitive proof of identity, because different compounds can sometimes have similar retention times. Confirmation should be performed using an additional orthogonal technique such as:

- HPLC-DAD/UV spectral comparison
- LC-MS or LC-MS/MS
- Other suitable spectroscopic techniques

Note: The suspected compound will be tentatively identified based on comparison of its HPLC retention time with an authentic reference standard and the corresponding increase in peak response following standard spiking.

Spiked Standard Method (Quantitative Analysis)

Purpose

To provide a standardized procedure for **quantitative estimation of a target compound in a sample by HPLC/PDA using the spiked standard method**. This guideline is intended for facility users and is applicable when an authentic reference standard of the analyte is available.

2. Principle

The sample is analyzed by HPLC/PDA before and after addition (**spiking**) of a known amount of the target standard. The increase in the analyte peak area after spiking is used to determine the amount of analyte present in the original sample.

Sample and Standard Requirements

- Submit 2-5 mg clearly labelled sample.
- The sample should be completely soluble in the mobile phase or compatible RP HPLC solvents.
- Provide an authentic reference standard (2-3 mg) of the targeted compound, preferably with documented purity.

3. Procedure

Step 1 – Prepare Sample Solution
Prepare the sample at a known concentration and filter through an appropriate membrane filter (e.g., 0.22 or 0.45 µm).

Step 2 – Prepare Standard Solution
Prepare a reference standard solution at a accurately known concentration.

Step 3 – Analyze Unspiked Sample
Inject the sample solution and record:

- Retention time (RT)
- Peak area
- PDA spectrum/peak purity, where applicable.

Step 4 – Prepare Spiked Sample
Add a known volume of the standard solution to a known volume of the sample solution. Mix thoroughly.

Step 5 – Analyze Spiked Sample
Inject the spiked sample under exactly the same HPLC conditions and record the target peak area.

Step 6 – Calculate Analyte Concentration

The concentration of analyte can be calculated from the increase in peak response produced by the known standard addition.

5. Basic Calculation

For a single-point spike, assuming detector response is proportional to concentration:

$$C_x = \frac{\Delta A}{A_s} \times C_s \times \frac{V_x + V_s}{V_x}$$

Where:

- C_x = concentration of analyte in the original sample
- $\Delta A = A_{spiked} - A_{sample}$
- A_s = peak area corresponding to the standard contribution
- C_s = concentration of reference standard
- V_x = volume of sample used
- V_s = volume of standard added

Important: If the standard and sample undergo different dilution factors, these must be incorporated into the calculation.

HPLC/PDA Fingerprinting of Phytochemical Extracts/Fractions

Protocol I: Preparation of 70% Ethanol Extract and Liquid–Liquid Fractions

1. Starting Material

Plant material: 250 g dry weight

Plant part used: Root / Leaves / Stem / Seeds / Bark / Whole plant / Flower

1. Dry the plant material completely and record the **dry weight (250 g)**.
2. Pulverize the material to a coarse/fine powder using a suitable grinder.
3. Store the powdered material in a clean, dry, airtight container until extraction.

2. Extraction with 70% Ethanol

Solvent: 70% EtOH in water (v/v)

1. Transfer **250 g powdered plant material** into a suitable extraction vessel.
2. Add approximately **2.5 L of 70% ethanol** (1:10, w/v).
3. Mix thoroughly and keep for **24–48 h** at room temperature with occasional stirring/shaking.
4. Filter through suitable filter paper or a Büchner funnel.
5. Repeat the extraction **2–3 times** with fresh 70% ethanol to maximize extraction.
6. Combine all filtrates.
7. Concentrate the combined extract under reduced pressure using a rotary evaporator at a temperature preferably **≤40 °C**.
8. Remove residual solvent under vacuum, if required.
9. Record the weight of the resulting **Aq-EtOH crude extract** and calculate the extraction yield.

3. Preparation of Aq-EtOH Crude Extract

1. Dissolve/suspend the dried crude extract in a minimum suitable volume of **water or aqueous ethanol**.
2. Transfer the solution/suspension to a separatory funnel.
3. Ensure that the extract is sufficiently aqueous to permit efficient liquid–liquid partitioning.

4. Liquid–Liquid Extraction

Partition the aqueous extract successively with solvents of increasing polarity.

Suggested sequence:

Aq-EtOH crude extract → n-Hexane fraction → Chloroform/DCM fraction → Ethyl acetate fraction → Aqueous fraction

For each solvent:

1. Transfer the aqueous extract into a separatory funnel.
2. Add an appropriate volume of the selected organic solvent.
3. Shake gently with frequent venting.
4. Allow the layers to separate completely.
5. Collect the organic layer.
6. Repeat the extraction **2–3 times** with fresh solvent.
7. Combine the corresponding organic layers.
8. Dry the organic fraction over anhydrous sodium sulfate, where appropriate.
9. Filter and concentrate under reduced pressure at a suitable temperature.
10. Record the weight of each dried fraction.
11. Store fractions in appropriately labelled containers, preferably at **4 °C or below**, protected from light.

5. Fraction Labelling

Fraction	Suggested Code
70% EtOH crude extract	Aq-EtOH-CE
n-Hexane fraction	HEX
Chloroform/DCM fraction	DCM/$CHCl_3$
Ethyl acetate fraction	EtOAc
Remaining aqueous fraction	AQ

6. Documentation

For every extraction, record:

- Plant name and voucher/specimen number
- Plant part used
- Collection location and date
- Starting dry weight: **250 g**
- Solvent and solvent volume
- Number of extraction cycles
- Crude extract weight
- Weight of each solvent fraction
- Percentage yield of crude extract and fractions
- Batch/extraction number
- Date and operator initials

Safety: Perform solvent extraction and concentration in an appropriate fume hood. Follow institutional chemical-safety procedures and consult the SDS for each solvent before use.