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BASIC CONSIDERATIONS FOR THE METHOD DEVELOPMENT IN HIGH PERFORMANCE LIQUID CHROMATOGRAPHY : A REVIEW

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ABSTRACT
The study's goal is to identify fundamental factors that should be taken into account while developing a high-performance liquid chromatography technology. Two sorts of samples can be distinguished based on how soluble their constituent parts. There are many different types of samples available. Since liquid form is easier to inject, all of them can be turned into liquid forms and they can be divided into two sorts based on the separation. By choosing an appropriate wavelength/lambda maximum at which the sample gives a strong peak with a gaussian distribution, the detection sensitivity is enhanced. Various pre-treatment techniques in the right solvent must be used on the sample during sample preparation. Desired peak should be completely separated from neighbouring peak. Sensitivity is the ability to detect low concentrations of analytes, while specificity is the ability to distinguish between different analytes.

Keywords: High performance liquid chromatography, Gaussian distribution, Pre treatment techniques, Right solvent, Wavelength Separation, Sensitive Detection.

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INTRODUCTION

HPLC is a chromatographic technique that can separate and analyze various compounds. It is widely used in different fields and applications. The development of an HPLC method involves several steps and considerations. The experimental conditions, such as the mobile phase, the stationary phase, the flow rate, the temperature and the detection wavelength can affect the quality of the results obtained by the HPLC method. The method development should follow a systematic and logical approach. The optimization of the parameters is crucial for achieving the best separation and resolution of the analytes. The accuracy and precision of the HPLC method can be enhanced by using ultraviolet (UV) detectors. This review article discusses the basic considerations to improve the performance of the HPLC method.

SAMPLE PREPARATION

Types of Samples: The samples can be either organic (with biological components) or inorganic and they can be classified into different states, such as solids, semi-solids (with creams, gels, suspensions, colloids), liquids, and gases. Gas chromatography is usually the preferred method for analysing gaseous samples, but HPLC can be more suitable for gaseous samples that are sensitive, unstable, or likely to stick to metal surfaces. **Liquid samples are easy preparations for HPLC than gases or solids.**

Typical Sample Pre-treatment Methods for Gases, Liquids, and Suspensions

Solid-phase Trapping: The gaseous sample is passed through a tube containing an adsorbent material (for example, silica gel, activated carbon). The analytes that are trapped on the adsorbent are then removed with a powerful solvent

Liquid trapping: Gaseous sample is passed through solution.

Solid-phase extraction: Liquid is passed through solid phase, which selectively removes analyte

Liquid-liquid extraction: Sample is partitioned between two immiscible phases.

Dilution: Sample is diluted with solvent compatible scope or reactive acid anhydrides.

Filtration: Particulates should be removed by filtration, centrifugation, and sedimentation. Paper filtration is a relatively straightforward technique. Filters in the range of 0.25 to 2-µm nominal porosity are recommended.

Extraction: (A) Liquid–Liquid Extraction, (B) solid-phase Extraction.

RP-SPE can often eliminate column killers, which are substances that can clog or damage the HPLC column. These include hydrophobic substances (such as fats, oils, greases), polymeric materials, and particulates.

Solutions ready for injection need to be prepared. **Some samples need sample pre-treatment to eliminate interferences or prevent damage to the column or equipment.** HPLC is selected for separation and then the next step is to classify them whether they are regular (or) special. Regular samples are typical mixture of small molecules (<2000 Da) for the separation at the starting conditions it can be more or less standardized and they are of two types neutral (or) ionic. The sample is regular otherwise note down the special characteristics. From starting to ending the

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conditions remains same. Experimental runs can be carried out and one should verify that each chromatogram is reproducible. When altering the parameters sufficient time between method development experiments is required **to allow the column to adjust to the new mobile phase and temperature** and separation on prior experiments.

The goal of sample preparations is a sample aliquot that meets the following criteria:

- **Sample having minimal interferences, is safe for the column and matches the planned HPLC method.**
- **The accuracy, precision, and reliability of the results depend greatly on the steps such as sample collection, transport, storage, preliminary processing, laboratory sampling, and subsequent weighing and dilution.**

Sampling procedure

Sample collection: Use a valid statistical process to get a representative sample.

Sample storage and preservation: Store the sample in suitable inert, airtight containers sample

Preliminary sample processing: More efficient Preliminary sample processing **Sample pre-treatments such as Drying, Sieving, Grinding and so on.**

Weighing or volumetric dilution: Take necessary precautions **Use measured volumetric glassware for dilution.**

Other sample processing method: Solvent change, salt removal, drying, lyophilization etc.

Particulate elimination: Filtering, solid-phase extraction, spinning.

Sample extraction: Different methods for liquid and solid samples

Derivatization: Mainly used to improve analyte detection

SOLVENT STRENGTH

This parameter represent the strength of various solvents or solvent mixtures for NPC, which can be determined experimentally. B solvents are needed for NPC. The cyano column separates a two-compound sample with MTBE and hexane mixtures as mobile phase. The run time reduces from 8 min to 4 min as % MTBE increases from 3% to 12%. The Soczewinski equation controls the retention.

$\log k = c - n \log X_B$

Here, C and n are constants for a particular solute.

B- Solvent and column.

X_B is the mole fraction of B-solvent in the mobile phase.

N = is approx. to the number of polar, localizing groups in the solute molecule.

A more convenient form of Eq 1 which is of comparable reliability is

$\log k = cl - nl \log (\%B)$

Where cl and nl are also constant for a particular solute, B –solvent and column.

A large number of NPC studies that verify both above equations Experimental data points for a solute fall close to a straight-line plot of log k vs. log % B over a wide range in % B.

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Some NPC columns that are commonly used are cyano, silica, diol, & amino. The column strength can be compared as in the case of RPC separation: silica > alumina>>amine>diol> cyano. Basic compounds (such as amines, ethers, esters, ketones etc.) are more retained on amine & diol columns. Dipolar compounds (with chloro, nitro, nitrile substituents) have stronger retention on cyano columns than on amino /diol.

THE COLUMN

Recommended Column for Method Development is Silica-based bonded-phase columns as the separating medium for most HPLC methods. Other types of column packings are not needed for these separations.

How to Maintain the Column for Optimal Performance and Longevity

- Use columns that are well-packed.
- Avoid pressure changes and mechanical or thermal shocks.
- Use a filter and a guard column in-line.
- Clean the column often with a powerful solvent.
- Remove particulates and unwanted components from dirty samples before analysis.
- Strongly retained
- Choose a stationary phase that does not change with time or conditions.
- Use buffers that contain organic compounds when the pH is between 6 and 8.
- Keep the temperature of the column below 40 °C, unless the stationary phase is protected from hydrolysis at low pH.
- Maintain the pH of the mobile phase between 3.0 and 8.0 for most columns that are based on silica. 10. Add 200 ppm of sodium azide to the mobile phases and buffers that are aqueous.
- For storage and overnight, remove salt and buffers from the column and fill it with pure organic solvent (acetonitrile is preferred)

These are the column specifications that we suggest for most studies that aim to develop methods

- Column dimensions: 25 or 15 X 0.46 cm
- Support material: 5-µm silica spheres with pores
- Pore diameter: 80 to 100 Å (except for large molecules)
- Surface area of the particles: 150 to 350 m^2/g
- Coating on the particles: C18 or C8 (Reversed phase); CN or diol (Normal phase)

You can easily find columns with the above characteristics that have good performance. As you continue to develop your method, you may need to change the column shape, particle size, and other factors.

In Normal phase chromatography the stationary phase is more polar than mobile phase where as in Reserve phase chromatography the stationary phase is less polar than mobile phase. Regardless, sample retention in NPC increases as the polarity of the mobile phase decreases. It is opposite to the RPC that is sample retention in RPC decreases as the polarity of the mobile phase Increases

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Factors influencing separation

- **Resolution:** By the chromatographic graph the partial separation will be happens in the six different bands and then the band 1 & 4 are separated and 2,3,5,6 are partially overlapped. The value of Rs is low for the two bands that have a lot of overlap.

$R_s = 2(t_2-t_1)/ W_1+W_2.$

- The resolution between two adjacent bands have R_s=1. The ratio of the distance between the centres of the peaks to the average width of the bands is the resolution.

Effect of column plate number: Several factors to increase the column plate number

1) Well packed column
2) Longer columns
3) Lower flow rates
4) Smaller column packing particles
5) Higher temperature lower mobile phase viscosity
6) Smaller sample molecules
7) Minimum extra column effects.

How the sample volume affects the separation: Sample volume affects the separation of analytes in chromatography. The sample is dissolved in the mobile phase and injected as a plug with a volume of Vs. The baseline width of the plug is 4σ. The peak volume of an analyte with a small retention volume (Vc) is given by the equation:

$V=(4/3Vs^2+Vc^2)1/2$

For large values of Vc, the peak volume is approximately equal to:

$V\approx(Vs^2+Vc^2)1/2$

DETECTOR

Most commonly used universal Detectors in HPLC are (1). RI detector (Refractive index) and (2). ELSD (Evaporative light scattering detector).

Fluorescence detector: Can able to discriminate analyte from interference/background peaks

Electrochemical detector: Classification according to their operation (1). Direct-current amperometry (DCA) (2). Conductivity detector

The Electrochemical Detector can sense these types of compounds:

Oxidation	Reduction
Phenolic	Ketones
Oximes	Aldehydes
Dihydroxy	Oximes

Less Common HPLC Detectors

(1) Reaction detector: A module that performs a chemical reaction between the analyte(s) and a UV or fluorescence detector, after they pass through the column, to make them more detectable. Mainly used for low-level analysis.
(2) Radioactivity: Extremely sensitive and specific for samples that are radiolabelled.
(3) Infrared (IR): Used only with a limited range of organic solvents for analysis of synthetic polymers.
(4) Optical activity (polarimeter): Used to detect enantiomers.
(5) Low-angle laser light scattering: Used to calculate molecular wt of synthetic and biopolymers.

Sensitivity and Selectivity of Detector: The background of this experiment is to find solvent mixtures that have low absorbance at 200 nm (0.5 AU) for liquid chromatography. The aqueous phase consists of different mixtures of the following solvents:

- 0.2% acetic acid
- 0.4% trifluoroacetic acid
- 0-100% acetonitrile-water
- 0-26% methanol-water
- 0-28% isopropanol
- 0-20% tetrahydrofuran (THF)

To the aqueous phase, 25 mM of either sodium phosphate or potassium phosphate (pH 5) is added. Then, 250 mM of sodium chloride (NaCl) (pH 6.8) is added to the mixture. The final solution is the mobile phase for chromatography.

The maximum concentration of acetonitrile that can be used in the mobile phase for high-performance liquid chromatography (HPLC) is 50%. This is because acetonitrile has a high absorbance of 1.0 at 190 nm, which interferes with the detection of analytes. Below 200 nm, the only additives that can be used in more than 1 mM concentrations are aliphatic amines and phosphoric acid. These additives have little absorbance and can change the pH and the polarity of the mobile phase.

Normal-phase chromatography: How the wavelength affects the UV absorbance of solvents in normal phase.

Absorbance (A) at Wavelength (nm) Indicated

Solvent	200	210	220	230	240	250	260
Ethyl acetate	>1	>1	>1	>1	>1	1	0.10
Ethyl ether	>1	>1	0.4	0.2	0.1	0.1	0.05
Hexane	0.5	0.2	0.07	0.03	0.02	0.1	0
Methylene chloride	>1	>1	>1	1.4	0.09	0	0

The illustration shows how to measure the signal (S) and the noise (N') of an analyte peak in chromatography. The signal is the absorbance of the peak after subtracting the baseline. The noise

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is the width of the baseline at the same height as the peak. The baseline noise has two sources: A long-term variation due to temperature changes and a short-term fluctuation due to stray light. The rise time of the detector affects the noise level. A shorter rise time (0.1 s) makes the short-term noise more dominant, while a longer rise time (5 s) makes the long-term noise more dominant.
The precision of an assay depends on the signal-to-noise ratio (S/N') and the coefficient of variance (CV) of the analyte concentration. The signal-to-noise ratio is the ratio of the peak absorbance to the baseline width. The CV is the ratio of the standard deviation to the mean of the analyte concentration. By plotting the CV against the analyte concentration, we can see how the precision changes with the concentration. If the CV is constant, then increasing the S/N' will not improve the precision. However, if the coefficient of variance increases as the concentration decreases, then increasing the S/N' will improve the precision.

Improving the ratio of signal to noise for more accuracy:
The analyte concentration, the sample volume, and the applied voltage. Increasing these factors can improve the column efficiency (N), which is the number of theoretical plates. However, they can also reduce the column capacity (V), which is the ratio of the amount of analyte retained on the column to the amount of analyte in the mobile phase. Moreover, they can decrease the analyte stability and cause peak broadening (K), which is the ratio of the peak width to the retention time. Peak broadening reduces the resolution and the sensitivity of the analysis
The detector time constant ® or rise time (2r). This is the time required for the detector signal to reach 63.2% of its final value. Increasing r can eliminate the high-frequency noise, which is the short-term variation of the baseline due to stray light. However, increasing r can also cause peak distortion, such as broadening and tailing, which results in signal loss. As r increases from 0.1 to 1.0 s, the noise level increases by four times, while the peak height remains the same. When r increases to 4 s, the noise level decreases, but the optimal value of r is 2.0 s.
Noise: As the light intensity decreases, noise increases, and the detector lamp gets older, more high frequency noise can occur.

Detection Sensitivity affected by Rise Time

Rise time r(min)	Signal (peak height)	Noise	S/N ratio
0.1	46	11	4.2
1	46	3	15.3
2	43	2	21.5
4	39	2	19.5
5	38	2	19

Detection sensitivity increase by increase S/N ratio & decrease noise by increasing rise time. To reduce noise from late elutes, you can try these methods: Clean the sample before HPLC, clean the column with a strong solvent, use guard columns, elute with a gradient, or switch columns.
Detector Linearity: How to improve UV detection sensitivity (S/N'):

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1. Choose the wavelength that gives the highest signal (S).
2. Use the biggest sample volume that you can (S).
3. Make the sample more concentrated to increase the mass injected (S).
4. Make k as small as possible (but avoid baseline disturbance or overlapping peaks) (S).
5. Think about using a different detector that is not UV.
6. Make the detector time constant larger (N'). (To the best value)
7. Replace the old lamp with a new one (S, N').
8. Use a pulse damper to reduce pump noise if needed (N').
9. Make sure the A and B solvents have the same UV absorbance if you mix them on-line (N').
10. Reduce late elutes by cleaning the sample, using gradient elution, or switching columns (N).

PROPERTIES OF CHROMATOGRAPHY IN NORMAL PHASE

Advantages:

1. You can change the separation selectivity a lot by changing the mobile phase or the column packing.
2. The columns are very stable with mobile phases that do not have water.
3. Normal phase solvents can dissolve many organic compounds better.
4. The pressure drop is smaller because the solvents have lower viscosity.
5. It is good for samples that may break down in water solutions.

Disadvantages :

1. In RPC ionic samples are more easily separated .
2. Controlling solvent strength can be Less predictable and more tedious than in RPC.
3. When compared with RPC Column plate numbers less in NPC.
4. At higher room Temperatures Lower-boiling point solvents are more prone to evaporation and bubble Formation.

5. Unmodified Silica

(a) Based on water uptake by The column packing Retention can be variable.

(b) Because of solvent gradient elution may not be practical.

ION PAIR CHROMATOGRAPHY

The ions interact compensating the ionic character of the analyte, & the separation is typically done using a reverse phase HPLC column.

pH & ion pairing: The acidic sample RCOOH and a positively charged ion pair reagent Eg: Tetrabutylammonium, TBA+ in ion pair reagent is added to the mobile phase at low PH, the non ionized RCOOH molecule is strongly retained vs the ionized acid RCOO- less retained, so retention vs PH conditions exhibit. Maximum retention occurs at low PH and minimum retention at high PH.

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ION EXCHANGE CHROMATOGRAPHY

Ion exchang chromatography separates ions and polar molecules based on their affinity to the ion exchanger. Detectability: - Many inorganic salts are not easily detected using typical HPLC detectors. the wide spread use of ion exchange chromatography for such samples. Organic ions with poor UV absorptivity are also candidates for this approach Eg:- alkyl amines or sulfonates.

Basics of Retention: Columns used for ion exchange are characterized by the presence of charged groups covalently attached to the stationary phase :-

1. Anion -Exchange columns carries positive charge usually a quaternary ammonium / amine group.
2. Cation – Exchange columns carries negative charge usually a sulfonate or carboxylate. The effect of the counterion concentration on retention can be generalized for a sample ion of charge z & a univalent counterion.

K = constant/ (counterion concentration) z

pH Effects: IEC is typically used for acidic or basic samples the effects pH on retention in ion exchange to be an increase in pH leads greater sample ionization & retention in anion exchange separations of acids while a decrease in pH favors the retention of bases by cation- exchange HPLC

Salt / Buffer Type: Different mobile phase anions or cations are retained more or less strongly in ion exchange can speak of strong or weak ionic displacers or counterions the relative strength of different displacers in Anion – exchange chromatography.

$F^- < OH^- <$ acetate$^- < Cl^- < SCN^- < Br^- < CrO^{4-} < NO^{2-} < I^- <$ Oxalate$^{2-} < SO_2^{2-} <$ citrate^{3-}.

Organic Solvent: The addition of an organic solvent to the mobile phase results in decreased retention HPLC solvents such as methanol or acetonitrile are also often exchange to create changes in selectivity.

GRADIENT ELUTION

Gradient elution separation uses the same mobile phase composition but gradually change their ratios throughout the separation in the gradient elution. Gradient elution cannot be used with some HPLC detectors but we see in UPLC detectors Baseline problems are more common with gradient elution, & solvents must be of higher purity.

Applications of Gradient Elution: Samples with a wide k range there is no isocratic conditions result in $0.5 < k < 20$ Samples containing late -eluting interferences that can either foul the column & dilute solution of the sample dissolved in a weak solvent.

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Gradient Elution for Method Development: When starting HPLC method development for a sample whose composition is undefined, there gradient elution best to separate the components from the undefined sample composition, the technique is carried out until the elution of last eluted. The sample's retention range can be estimated from this gradient run, which also helps to choose between isocratic and gradient elution for further experiments. Isocratic elution is faster and more suitable for samples that are too weakly or strongly retained in reversed phase HPLC. A standard set of conditions for isocratic elution is a 15 × 0.46-cm column, 2 ml/min flow rate, and a constant acetonitrile percentage that matches the sample's retention time in the gradient run. If no sample peaks are observed, either the detector response is poor or the sample is too hydrophilic for reversed phase separation.

The gradient run lasts for 60 minutes with a flow rate of 2 ml/min. The sample is separated by reversed phase HPLC. If the detector does not show any sample peaks, it means that either the detector is not working properly or the sample is not compatible with reversed phase HPLC.

Estimating the Best Isocratic & Gradient Elution: For a sample with a molecular weight below 2000 Da, we can estimate the optimal initial and final %B values from the retention times of the first and last bands in a gradient run. We assume that the first band elutes at 10 min and the last band elutes at 40 min. We can use the retention time of the last band in the gradient run to find the best isocratic elution conditions that will give a desired value of K for the last band. For a 15 × 0.46 -cm column, a flow rate of 2 ml/min, and a gradient time of 60 min, the predicted mobile phase composition for isocratic elution is 37% acetonitrile for the last band. We can check if isocratic separation is possible by comparing the retention times of the other bands in the gradient and isocratic runs.

TRa (min)b	1< k<10	0.5<k<20
<1.5 C	c	
2	8	17
3	12	21

Principles of Gradient Elution: As the separation progresses, the mobile phase strength increases in gradient elution. This causes the retention factor k of each band to decrease as it travels through the column. When the separation starts, the percentage of organic solvent (%B) is low and k for band X is high. After some time, %B increases and k for band X becomes low enough for it to enter the column. As %B continues to increase, k for band X decreases further and X moves faster. It reaches the end of the column and is detected as a peak in the chromatogram.

Gradient Vs Isocratic Elution: The mobile phase composition does not change in isocratic separation and each band has the same retention factor k throughout the column. The retention

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factor k is constant for a given band in isocratic separation. The equation for k in gradient elution is:

$$K^* = 87\ tG\ F/Vm`\ (\%\ B)$$

where tG is the gradient time, F is the flow rate, Vm′ is the column dead volume, and %B is the percentage of organic solvent.

QUANTITATION (INCLUDING TRACE ANALYSIS):-

Accuracy: Accuracy is defined as how closely a measured value resembles its true value. But the true value is different from technique to technique. It helps for proper calibration & minimising the source of errors.

Precision: It is defined as reproducibility of multiple measurements of a single sample. It gives results by using different instruments, analysts, sample preparations, laboratories & so on.

Linearity: By the calibration plot of responses Vs concentration gives straight line to detect the linearity. Y=mx+b. In UV detector responses the beer's law is used for the linearity. B⊇0 shows linear responses and b≠0 is non linear response.

Limits of Detection: It is also known as minimum detectable amount of analyte in this the smallest concentration is used for the detection. It related to signal to noise ratio it can have 3:1 for this noise is 10 units & signals is 30 units. It can done with three methods. These are S/N ratio, level of precision & baseline noise by Gaussian distribution, higher concentration is needed for quantitation.

Peak Height: To measure the responses from the detector to obtained the peak height of signal. Mainly it's used for trace analysis. The baseline contain long term noises. Tangent skimming happens on the small peaks on the tailing edge of a large preceding peak. Peak height is important for quantitation.

Peak Area: It is most widely used technique in HPLC. In this they are 3 conditions. They are

(1) To establish the baseline by short- or long-term noise.
(2) From the beginning to the end of the peak. It is difficult for non-symmetrical or tailing peak, leading to inaccurate quantitation.
(3) It uses the datapoints. Points for Gaussian peak & 32 points for non-gaussian peaks. 15 points are taken in this 3 to 5 points/ sec and column has 3μm.

The particle run measures the efficiency of the column. The efficiency is higher for symmetrical peaks than for asymmetrical peaks. The efficiency range is from **99.6% to 99.9%** for symmetrical peaks and from **92.3% to 97.8%** for asymmetrical peaks.

Quantitation Methods: There are three methods to calculate the normalized peak area in chromatography. They are:

1. External standard
2. Internal standard
3. Method of standard addition.

Normalized peak area: The significant peaks in the chromatograms can causes the total peak area. The individual peak shows the area percent is called normalized peak area. In this methos there is no comparison will be done at any peak in chromatogram. It is widely used method.

External standard calibration: This method uses a known concentration of a pure standard to compare with the sample peak area. The ratio of the sample peak area to the standard peak area gives the normalized peak area.

Internal standard calibration: This method adds a known amount of a different compound to the sample and the standard. The ratio of the sample peak area to the internal standard peak area gives the normalized peak area.

Standard addition method: This method adds increasing amounts of a pure standard to the sample and measures the peak area of each mixture. The normalized peak area is obtained by plotting the peak area versus the standard concentration and extrapolating to zero concentration.

Sources of Error in Quantitation: For good accuracy it certain:
1. A representative sample.
2. Minimum overlap of bands or interferences.
3. Good peak shape.
4. Accurate calibration with purified standards.
5. Proper data handling, including integration.

For good precision a method depends on:
1. Sample preparation technique.
2. Instrument reproducibility, including injection technique.
3. Acceptable S/N ratio for the peak of interest.
4. Good peak shape.
5. Proper data handling, including integration.
6. Method of quantitation or calibration.

Chromatographic Effect: It can show the sources of error for quantitation in HPLC.
1. Resolution between critical peak points of >1.5(preferably >2.0)
2. Reasonable retention for all peaks (0.5 <K<20 preferably 1<K<10)
3. Peak signal to noise (S/N) ratio>50 for quantitation.
4. Reproducible separations, day to day; different columns, instruments soon.

Data System Effects: Good quantitation results depend upon
1. How the system filters or average short term noise.

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2. The number of points/sec of data collected.
3. Data collection parameters specified.
4. The computer algorithms used to process the data.

Trace Analysis: HPLC is a powerful technique & it is used for the trace analysis of components. Reasons for using HPLC: -

1. High resolving power for accurate measurements.
2. Sensitive & selective detection often available.
3. Minimal pre treatment for some samples.
4. Original samples sometimes can be preconcentrated for higher sensitivity.

Column Resolution: Desired peak should be completely separated from neighbouring peak. When two peaks become closer the measurements of trace compounds should be accurate in a major constituent.

Sample Injection: For trace analysis, the components need to have maximum sensitivity and the sample volume should be as large as possible for injection. However, overloading the sample can cause distorted peaks with less increase in peak height. The sample volume should be less than one-fifth of the volume of the first peak of interest. Overlapping of peaks can also occur. For a column size of 15x0.46 cm, the sample volume should be between 50 and 500 µl.

Detection: The detectors are mainly used for selectivity and sensitivity in trace analysis. They respond to the signal-to-noise ratio (S/N). In HPLC trace analysis, the detection systems include UV spectrophotometry, fluorimetry, electrochemistry, and mass spectrometry. Some detectors have variable wavelength UV capability.

Calibration: The various methods are there for calibrating the compounds and mainly they are peak height is preferred for the strong potential for accuracy but it is used very less than peak area measurements. Peak height is more convenient for trace analysis methods

General Strategy: Trace analysis is a method that requires a balance between sensitivity and specificity. Sensitivity is the ability to detect low concentrations of analytes, while specificity is the ability to distinguish between different analytes. Isocratic elution is usually preferred for trace analysis, as it provides constant retention factors and peak shapes. However, gradient elution can also be used in some cases, such as when the analytes have a wide range of polarity or when the sample matrix is complex.

Limitations:

- Major limitation of gradient elution for trace analysis is artifacted peaks that often occur in blank gradient.

- Gradient operation usually results in longer analysis times, mainly because the column must be re-equilibrated before the next run.
- Some detectors can't be used with gradient elution.
- Gradients can causes strongly sloped baselines or less stable detector baselines.

A sequences of typical trace analysis can involve following steps: -

1. Selectively isolate the analyte by liquid-liquid or Solid phase extraction usually into a volatile organic solvent. (eg., Dichloromethane, methyl-t-butyl ether).
2. Concentration the sample by solvent evaporation.
3. Select a 7.5x10.46 cm column with 3 or 3.5 μm particles & a C18 or C8 bonded stationary phase.
4. Start with a UV detector, to detect the components.
6. To achieve the desired resolution from potential interfering chemicals, create the best separation utilising a reasonably high analyte concentration.
7. Optimize the sample injection volume & construct a peak height calibration for the concentration range of interest.
8. If sensitivity is insufficient, use an electrochemical or fluorometric detector, if possible.

Implementation of Design Expert (V 12) software for HPLC Method Development

Various steps involved in it:

- If we go for Response surface methodology
- Central Composite Design
- Take the response from the excel sheet
- Input the parameters in a CCD page
- Click on the OPTION button
- Here No. of forms are: -Renewable, Spherical, Orthogonal quadratic, Radical, Face central, others
- In this if we have select face central then some response will come
- Then Click on NEXT button
- Select the response here
- Here insert the parameters that what responses we want
- Click on FINISH button
- Then the matrix results will appear
- Remaining values will be taken from excel sheet
- Analysis of values
- Models should be selected here
- Then the models are: Transformation, Fit summary, fx submodel, ANOVA, Diagnostic, Model graph
- By the ANOVA model we can check that all values in a right way or not. It is software that it can easily helpful for analysis for drugs

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- Then next diagnostics in this the significant (or) accuracy will check here
- Next model graphs can have different model graphs. They are: 2d, 3d, interaction, predicted vs actual (RS model)
- In the report we can find the all values what we want by this method
- Optimization
- Numerical Optimization
- Select the criteria first
- Then click on SOLUTIONS for next
- Best solutions/ desired solutions will be shown here
- Then it can also be seen in graphical representation
- Bar graphs, Individual graphs and 3D desired graphs will also come.

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