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HPLC Technique used in Food Analysis – Review

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***Abstract* – HPLC is the dominant separation technique in food because it results in highly efficient separations and in most cases provides high detection sensitivity. Most of the food in multi component dosage forms can be analyzed by HPLC method because of the several advantages like rapidity, specificity, accuracy, precision and ease of automation in this method. HPLC methods development and validation play important roles in new discovery, development, and manufacture of food. This review gives information regarding various stages involved in development and validation of HPLC method, and knowing characteristics like Accuracy, specificity, linearity, range and limit of detection, limit of quantification.**

***Keywords* – HPLC, Chromatography, Food Analysis, Selection, Effect.**

I. INTRODUCTION

High performance liquid chromatography (HPLC) is an integral analytical tool in assessing product stability. HPLC methods should be able to separate, detect, and quantify the various food-related degradants that can form on storage or manufacturing, plus detect and quantify any food-related impurities that may be introduced during synthesis [1] High-pressure liquid chromatography (HPLC) is a means of separating constituent chemical compounds in a mixed solution in a chromatographic column. They can then be purified (preparative HPLC) or identified and quantified (analytical HPLC) [2] HPLC is now one of the most powerful tools in analytical chemistry as it has the ability to identify, separate and quantitate the compounds that are present in any sample that can be dissolved in any liquid. Today, trace concentrations of compounds as low as parts per trillion [ppt] may easily be identified. HPLC can be, and has been, applied to just about any sample, such as food, pharmaceuticals, forensic samples, nutraceuticals, cosmetics, industrial chemicals and environmental matrices [3] High Performance Liquid Chromatography (HPLC) was derived from the classical column chromatography and, is one of the most important tools of analytical chemistry today. The principle is that a solution of the sample is injected into a column of a porous material (stationary phase) and a liquid (mobile phase) is pumped at high pressure through the column. The separation of sample is based on the differences in the rates of migration through the column arising from different partition of the sample between the stationary and mobile phase. Depending upon the partition behaviour of different components, elution at different time takes place. The technique, chromatography was originally developed by the Russian botanist M. S Tswett in 1903 [4] HPLC is more versatile than gas chromatography since (a) it is not limited to volatile and thermally stable samples, and (b) the choice of mobile and stationary phases is wider. High resolution. Small diameter (4. 6 mm), stainless steel, glass or titanium columns. Column packing with very small (3, 5 and 10 μm) particles. Relatively high inlet pressures and controlled flow of the mobile phase. Continuous flow detectors capable of handling small flow rates and detecting very small amounts. Rapid analysis [5] .

II. PRINCIPLE OF CHROMATOGRAPHY

To understand the principles of HPLC we needs to know the basic operation, elution processes and physical and chemical parameters of the technique and the selection of detectors for detecting the analyte and using in food. Chromatography can be simply defined as the process of separation of the individual components of a mixture based on their relative affinities towards mobile phases and stationary phases. The samples subjected to flow by a mobile liquid phase through the stable stationary phase. The sample compounds are separated into individual components based on their relative affinity towards the two phases during their travel. The sample compound with the greater affinity to the stationary layer will travel slower and for a shorter distance in comparison to compounds with less affinity which travel faster and for a longer distance [3] There are two principle stages in HPLC analysis: separation, in which the sample is resolved into its constituent compounds, and detection, where these compounds are identified and quantified The sample to be analyzed is introduced into the mobile phase, which is continuously pumped through the analytical column under high pressure, without disrupting the flow or changing the pressure. This is achieved using a high-pressure switching valve which may be a manual valve, where the experimenter "injects" each individual sample into the fluid stream, or it may be an auto injector, where the valve is incorporated in an auto sampler, such that batches of samples can be injected without the need for the experimenter to be present. The latter approach has advantages where large numbers of samples are to be analyzed but can have disadvantages in the amount of sample required to make an injection. If using an auto

injector, it is wise to include a chilled sample tray, to reduce degradation of the sample while waiting to be injected [2]

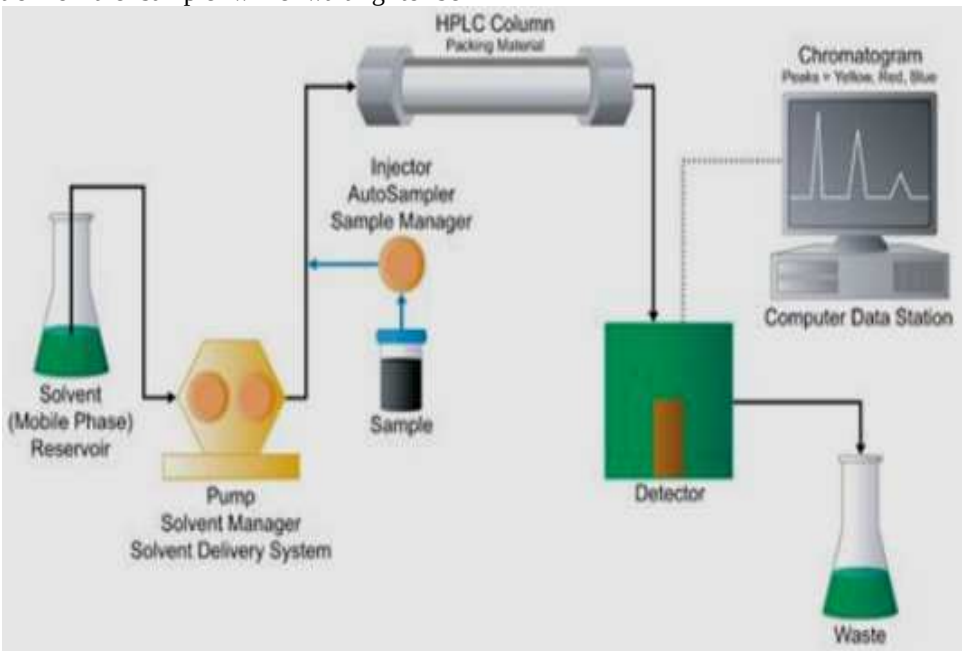

Fig. 1. Basic instrumentation of HPLC [3]

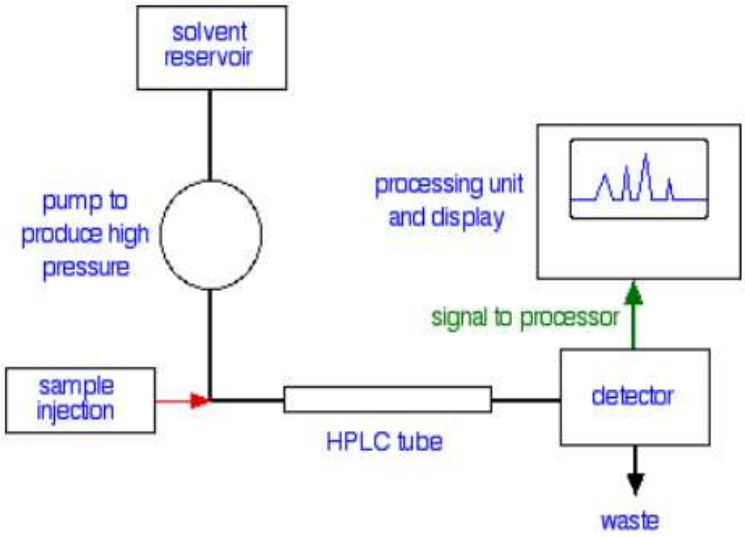

Fig. 2. A flow scheme for HPLC [3]

III. HPLC Conditions

A buffer is a partially neutralised acid which resists changes in pH. Salts such as Sodium Citrate or Sodium Lactate are normally used to partially neutralise the acid. Buffering Capacity is the ability of the buffer to resist changes in pH (i) Buffering Capacity increases as the molar concentration (molarity) of the buffer salt/acid solution increases. (ii) The closer the buffered pH is to the pKa, the greater the Buffering Capacity. (iii) Buffering Capacity is expressed as the molarity of Sodium Hydroxide required to increase pH by 1. 0. Consideration of the affect of pH on analyte retention, type of buffer to use, and its concentration, solubility in the organic modifier and its affect on detection are important in reversed-phase chromatography (RPC) method development of ionic analytes. An improper choice of buffer, in terms of buffering species, ionic strength and pH, can result in poor or irreproducible retention and tailing in reverse-phase separation of polar and ionizable compounds [5] During initial method development, a set of initial conditions (detector, column, mobile phase) is selected to obtain the first “scouting” chromatograms of the sample. In most cases, these are based on reversed-phase separations on a C18 column with UV detection. A decision on developing either an isocratic or a gradient method should be made at this point [6]

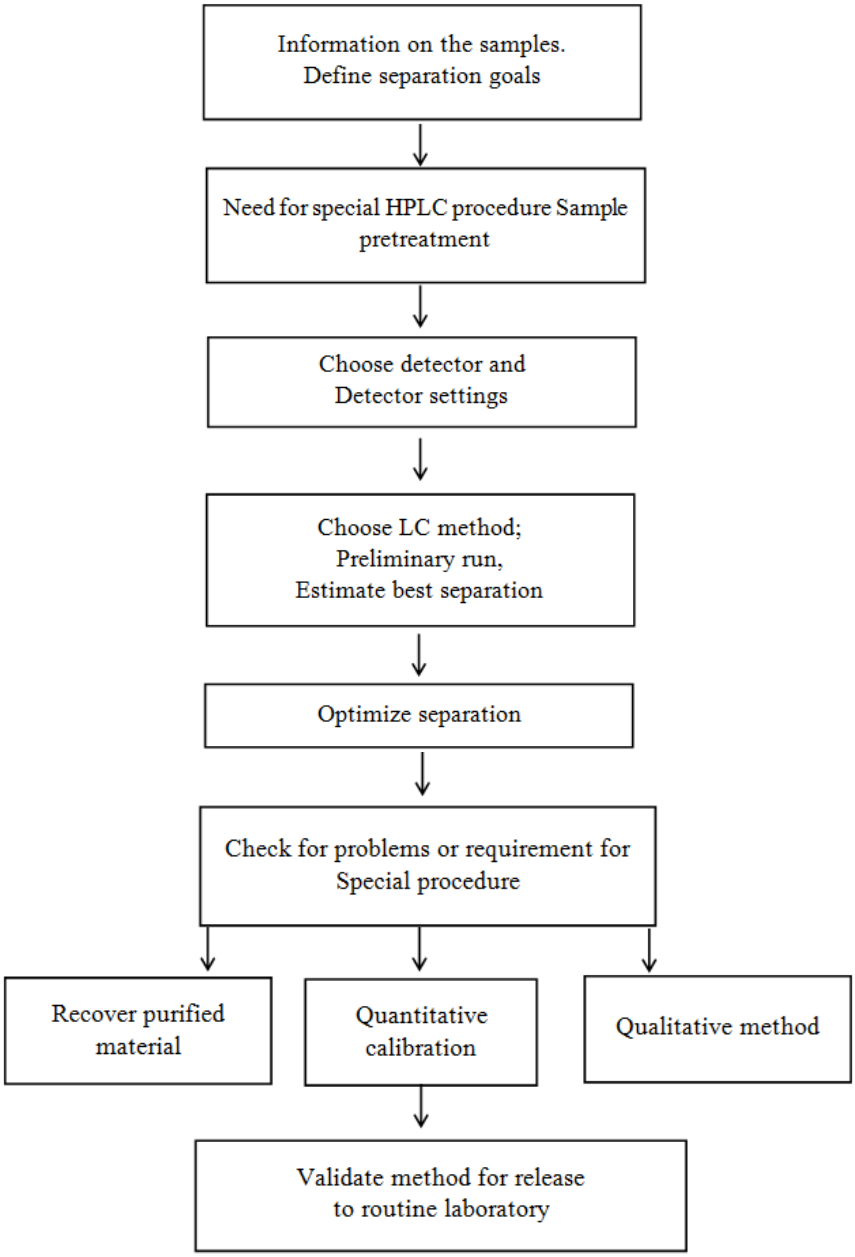

Fig. 3. Steps involved in HPLC Method development [6]

A. Effect of pH on Analyte Ionization

The primary mechanism of retention in RP chromatography is hydrophobic interaction. Ionizing compounds will cause them to behave as more polar species, and reduce their hydrophobic interaction with the stationary phase, leading to decreased retention. The ionization state of a molecule will be determined by the pH of the mobile phase and therefore pH of mobile phase will dictate the retention behavior of analytes with ionizable functional groups [3]

B. Effect of Temperature

Temperature conditions in HPLC method development present a challenge because it can have unpredictable effects on selectivity. The use of elevated temperatures will:

1. Reduce mobile phase viscosity and back-pressure. This can allow you to operate at higher flow rates or to use longer columns/smaller particle sizes.
2. Reduce elution time.
3. Improve method reproducibility (as opposed to operating at room temperature).

However, it is impossible to determine if the use of elevated temperatures will help or hinder a specific separation because for complex separations, improvements in one portion of the chromatogram are almost always accompanied by disimprovement in another part of the same chromatogram [7]

C. Effect of Retention Time

The time taken for a particular compound to travel through the column to the detector is known as its retention time. Retention time is measured from the time at which the sample is injected into the system to the point

at which the display shows a maximum peak height for that compound. Different compounds have different retention times. The retention time for a particular compound will vary depending on:

- the pressure used (because that affects the flow rate of the solvent)
- the nature of the stationary phase (material and particle size)
- the exact composition of the solvent
- the temperature of the column.

If you are using retention times as a way of identifying compounds conditions have to be carefully controlled [3]

D. Effect of Internal Diameter

The internal diameter (ID) of an HPLC column is an important parameter that influences the detection sensitivity and separation selectivity in gradient elution. It also determines the quantity of analyte that can be loaded into a column. Larger columns are usually seen in industrial applications such as the purification of a drug product for later use. Low – ID columns have improved sensitivity and lower solvent consumption at the expense of loading capacity. Larger – ID columns (over 10 mm) are used to purify usable amounts of material because of their large loading capacity. Analytical scale columns (4. 6 mm) have been the most common type of columns though smaller columns are rapidly growing in popularity. Analytical scale columns are used in traditional quantitative analysis of samples and often use a UV – Vis absorbance detector. Narrow-bore columns (1-2 mm) are used for applications when more sensitivity is desired either with fluorescence detection, UV – Vis detectors or with other detection methods like liquid chromatography – mass spectrometry. Capillary columns having a size under 0. 3 mm are used most exclusively with alternative detection means such as mass spectrometry. These columns are usually made from fused silica capillaries, rather than the stainless steel tubing that are employed by larger columns [8]

E. Effect of Particle Size

Most traditional HPLC is performed with the stationary phase attached to the outside of small spherical silica particles. These silica particles come in many sizes with 5μm beads being the most commonly used. The smaller particles usually provide more surface area and better separations but the pressure required for the optimum linear velocity increases by the inverse of the particle diameter squared. This implies that changing to particles that are half as big while keeping the size of the column the same will definitely double the performance but also increase the required pressure by a factor of four. Larger particles are used in preparative HPLC where column diameters are in range of 5 cm to >30 cm and for non-HPLC applications such as solid – phase extraction [9]

F. Effect of Pore Size

Many stationary phases are porous to provide greater surface area for the solvent. A small pore will provide greater surface area while a larger pore size has better kinetics, especially large analytes are used. Example, a protein which is only slightly smaller than a pore might enter the pore but does not easily leave once inside [8 -10]

G. Effect of Pump Pressure

Pumps vary in capacity of pressure but their performance is measured on their ability to yield a consistent and reproducible flow rate. Pressure may reach as high as 40 MPa (6000 lbf/in2 or about 400 atm). Modern HPLC systems have been improved to work at much higher pressure and therefore are able to use much smaller particle sizes in the columns (<2 μm). These "Ultra High Performance Liquid Chromatography" systems or RSLC/ UHPLCs can work upto 100 MPa :15000lbf/in2 or 1000 atm. [11] .

H. Effect of Solvent

The elution strength of a given solvent is determined by its hydrophobicity but the selectivity of a solvent is determined by its polar characteristics. Heptane and hexane have the same elution strength but different selectivity. For example, Methanol is a strong proton donor and a strong proton acceptor in hydrogen bonding. Acetonitrile has a dipole moment but is only a very weak proton acceptor in hydrogen bonding. Tetrahydrofuran accepts a proton in hydrogen bonding but cannot donate a proton [12]

IV. Chromatographic Separation

Separation is achieved in the analytical column and is dependent on the interaction of the constituent components in the sample with the mobile and stationary phases, which can be manipulated using different choices of solvents and column packing. These interactions are dependent on the size, the ionization, the polarity, and the stereoisometry of the molecule [2] . In RP-HPLC, separation is achieved by a partition of the compounds in the sample between the hydrophobic (nonpolar) stationary phase (the column packing material) and the hydrophilic (polar) mobile phase (the buffer) and is dependent on a compound's relative affinity for each: this in turn is dependent on the hydrophobic/hydrophilic nature of the compound itself. Generally, compounds that are more hydrophilic, and so more similar to the mobile phase, will pass through the column more quickly than those which are more hydrophobic, and so more like the stationary phase [13] The mobile phase in PR-HPLC is based on an aqueous (polar) solution, typically also containing a proportion of an organic solvent (e. g., methanol or acetonitrile). The organic content of the mobile phase is an important determinant of retention in the column. Retention will be greater (i. e., longer retention times) when mobile phases of low organic content are used, while higher organic content will result in less retention in the column and shorter retention times [2]

In RP-HPLC, a major factor in determining the retention of a compound in the column is the ionization of the compound, since the more polar a compound is, the less it will be retained in the column, and so the more quickly it will pass through. Therefore, the degree of ionization of analytes will have a major impact on the speed they pass through the column. This means that the pH at which the chromatography is carried out is a crucial factor in determining the separation. At low pH, acidic molecules

will be essentially unionized and so will be retained, while basic molecules will essentially be ionized and will pass through the column quickly. At high pH, the opposite will be true: unionized basic molecules will be retained, and charged acidic molecules will not (Lim 1986). Ion-pair HPLC may be seen as a hybrid of RP-HPLC and ion-exchange HPLC. An ion pairing agent, a molecule with one nonpolar terminal, which binds to the stationary phase, and a polar (ionized) terminal which is in contact with the mobile phase, is used. The principle effect of the ion pairing agent is to increase the retention in the column of compounds in the sample with an ionic charge opposite to that of the ion pair (since opposite charges attract): it may also have a small effect of decreasing the retention of similarly charges molecules, although the effect is minimal, and is of little use in changing separation characteristics [13- 14]

From this it can be seen that adjusting the pH, and the use of ion-pairing agents have differential effects on the retention times (i. e., they have different effects on different compounds), whereas changing the organic content of the mobile phase affects the retention of all compounds in an indiscriminate manner. For many separations, it is adequate to use an isocratic buffer system, that is, continuous perfusion with a single buffer. One drawback with this approach is that, in order to achieve good separation of compounds which elute more quickly from the column, those which elute more slowly are often retained for an unacceptably long time. This has two adverse effects: first, it can make the run time excessively long and therefore reduce the throughput of samples to an unacceptably low level, and second, the longer the peak is retained on the column, the wider and lower the peak appears, such that small peaks with long retention times may become impossible to measure [2]

To overcome this problem, gradient elution is often used. For this type of elution, the content of the mobile phase changes steadily over the duration of a chromatographic run, such that the strongly retained components of the sample elute more quickly, while the separation of the least retained compounds is maintained. The two most common gradient elutions used are a pH gradient (i. e., the pH changes over the duration of the run) or an organic gradient (i. e., the organic content of the buffer changes over the duration of the run) [14]

Table 1. The main categories of separation types used for preparative or analytical HPLC [13]

Type	Mechanism	Uses
Adsorption	Separation based on differences between adsorption affinities of the sample components for the surface of the stationary phase (often alumina)	Mainly used for preparative or purification HPLC
Partition	Separation based on the differences between the solubility of components in the mobile and stationary phases. There are two distinct types of partition chromatography:	Reverse phase chromatography widely used for separating many different classes of chemical compounds. It is the most versatile and widely used analytical HPLC technique
	Normal phase — partition between a polar stationary phase and a nonpolar mobile phase	
	Reverse phase — partition between a nonpolar stationary phase and a polar mobile phase	
Ion exchange	Separation based on differences between ionic charge of sample components. A charged stationary phase attracts molecules of opposite charge, which are retained on the column. These are then eluted off the column by introducing another ion of similar charge. Either anion or cation exchange columns can be used depending on the charge of the compound to be separated	Mainly used for preparative and purification HPLC
		Principles of ion-pair chromatography can be incorporated into reverse phase chromatography, by the use of ion-pairing agents
Molecular exclusion	Separation based on the hydrodynamic volume of the molecule to be separated relative to the pores in the non-adsorbing stationary phase	Mainly used for purification
Affinity	Separation based on the unique and highly specific biological interaction between the analyte and a ligand (e. g., enzyme-substrate interaction; antigen-antibody interaction)	Mainly used for purification: for example, nucleic acid purification, protein purification, antibody purification
Chiral	Stereoisomers separated by using chiral phases	Separation of stereoisomers of a compound

A. Selection of Column

A column is of course, the starting and central piece of a chromatograph. A appropriately selected column can produce a good chromatographic separation which provides an accurate and reliable analysis. An improperly used column can often generate confusion, inadequate, and poor separations which can lead to results that are invalid or complex to interpret [15] The heart of a HPLC system is the column. Changing a column will have the greatest effect on the resolution of analytes during method development. Choosing the best column for application requires consideration of stationary phase chemistry, retention capacity, particle size, and column dimensions. The three main components of an HPLC column are the

hardware, the matrix, and the stationary phase. There are several types of matrices for support of the stationary phase, including silica, polymers, alumina, and zirconium. Silica is the most common matrix for HPLC columns. Silica matrices are robust, easily derivatized, manufactured to consistent sphere size, and does not tend to compress under pressure. Silica is chemically stable to most organic solvents and to low pH systems. One short coming of a silica solid support is that it will dissolve above pH 7. In recent years, silica supported columns have been developed for use at high pH. The nature, shape and particle size of the silica support effects separation. Smaller particle results in a greater number of theoretical plates, or increased. The nature of the stationary phase will determine whether a column can be used for normal phase or reverse phase chromatography. Normal phase chromatography utilizes a polar stationary phase and a non-polar mobile phase. Generally, more polar compounds elute later than non-polar compounds. Commonly used reverse phase columns and their uses are listed below. Propyl (C3), Butyl (C4), and Pentyl (C5) phases are useful for ion-pairing chromatography (C4) and peptides with hydrophobic residues, and other large molecules. C3–C5 columns generally retain non-polar solutes more poorly when compared to C8 or C18 phases. Examples include Zorbax SB-C3, YMC-Pack C4, and LunaC5. These columns are generally less stable to hydrolysis than columns with longer alkyl chains. Octyl (C8, MOS) phases have wide applicability. This phase is less retentive than the C18 phases, but is still quite useful for pharmaceuticals, nucleosides, and steroids [16] .

Selection of the stationary phase/column is the first and the most important step in method development. The development of a rugged and reproducible method is impossible without the availability of a stable, high performance column. To avoid problems from irreproducible sample retention during method development, it is important that columns be stable and reproducible [6]

The heart of a HPLC system is the column. Changing a column will have the greatest effect on the resolution of analytes during method development. Generally, modern reverse phase HPLC columns are made by packing the column housing with spherical silica gel beads which are coated with the hydrophobic stationary phase. The stationary phase is introduced to the matrix by reacted a chlorosilane with the hydroxyl groups present on the silica gel surface. In general, the nature of stationary phase has the greatest effect on capacity factor, selectivity, efficiency and elution. There are several types of matrices for support of the stationary phase, including silica, polymers, and alumina. Silica is the most common matrix for HPLC columns. Silica matrices are robust, easily derivatized, manufactured to consistent sphere size, and does not tend to compress under pressure. Silica is chemically stable to most organic solvents and to low pH systems. One shortcoming of a silica solid support is that it will dissolve above pH 7. In recent years, silica supported columns have been developed for use at high pH. The nature, shape and particle size of the silica support effects separation. Smaller particle results in a greater number of theoretical plates, or increased separation efficiency. However, the use of smaller particles also results in increased backpressure during chromatography and the column more easily becomes plugged [5]

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Selection of the stationary phase/column is the first and the most important step in method development. The development of a rugged and reproducible method is impossible without the availability of a stable, high performance column. To avoid problems from irreproducible sample retention during method development, it is important that columns be stable and reproducible. The separation selectivity for certain components vary between the columns of different manufacturer as well as between column production batches from the same manufacturer. Column dimensions, silica substrate properties and bonded stationary phase characteristics are the main ones. The use of silica-based packing is favored in most of the present HPLC columns due to several physical characteristics [17]

B. Selection of Detectors

HPLC detectors are important accessories of the HPLC instrument. This part of HPLC helps in detection and

identification of compounds in the sample injected. The detectors are designed to have certain properties like:
they should be inert (non-reactive) to the samples injected and the mobile phases passing through.

- they should be preferably non-destructive to the sample.
- should be able to produce quick and quantitative response.
- reliable, uniform and reproducible detection and analytic data.
- compatible with all types of compounds under testing.
- should have good sensitivity (ablility to detect compounds at very low concentration in the ranges below µg, ng, etc.) as the sample quantity may be lower in many cases [18]

Detector is a very important part of HPLC. Selection of detector depends on the chemical nature of analytes, potential interference, limit of detection required, availability and/or cost of detector. UV-Visible detector is versatile, dual-wavelength absorbance detector for HPLC. This detector offers the high sensitivity required for routine UV-based applications to low-level impurity identification and quantitative analysis. Photodiode Array (PDA) Detector offers advanced optical detection for Waters analytical HPLC, preparative HPLC, or LC/MS system solutions. Its integrated software and optics innovations deliver high chromatographic and spectral sensitivity. Refractive Index (RI) Detector offers high sensitivity, stability and reproducibility, which make this detector the ideal solution for analysis of components with limited or no UV absorption. Multi-Wavelength Fluorescence Detector offers high sensitivity and selectivity fluorescence detection for quantitating low concentrations of target compounds [5]

C. Selection Buffer

Choice of buffer is typically governed by the desired pH. The typical pH range for reversed phase on silica-based packing is pH 2 to 8. It is important that the buffer has a pKa close to the desired pH since buffer controls pH best at their pKa. A rule is to choose a buffer with a pKa value <2 units of the desired mobile phase pH [5]

D. Concentration of Buffer

Generally, a buffer concentration of 10-50 mM is adequate for small molecules. Generally, no more than 50% organic should be used with a buffer. This will depend on the specific buffer as well as its concentration. Phosphoric acid and its sodium or potassium salts are the most common buffer systems for reversed-phase HPLC. Sulfonate buffers can replace phosphonate buffers when analyzing organophosphate compounds [18]

Table 2. HPLC Buffers, pKa Values and Useful pH Range [5]

Buffer	pKa	Useful pH Range	UV cutoff
Ammonium acetate	4. 8 9. 2	3. 8-5. 8 8. 2-10. 2	205nm (10mM)
Ammonium formate	3. 8 9. 2	2. 8-4. 8 8. 2-10. 2	
Ammonium hydroxide/ ammonia	9. 2	8. 2-10. 2	
KH_2PO_4/K_2PO_4	7. 2	6. 2-8. 2	<200nm
KH_2PO_4/ phosphoric acid	2. 1	1. 1-3. 1	<200nm
Potassium Acetate/ acetic acid	4. 8	3. 8-5. 8	210nm(10mM)
potassium formate/ formic acid	3. 8	2. 8-4. 8	210nm(10mM)
Trifluoroacetic acid	<2	1. 5-2. 5	210nm(0. 1%)
Borate ($H_3BO_3/Na_2B_4O_7$ $10H_2O$)	9. 2	8. 2-10. 2	
Tri-K-Citrate/hydrochloric acid 1	3. 1	2. 1-4. 1	230nm(10mM)
Tri-K-Citrate/hydrochloric acid 2	4. 7	3. 7-5. 7	230nm(10mM)
Tri-K-Citrate/hydrochloric acid 3	5. 4	4. 4-6. 4	230nm(10mM)

E. Selection of Chromatographic Mode

Chromatographic modes based on the analytes molecular weight and polarity. All case studies will focus on reversed phase chromatography (RPC), the most common mode for small organic molecules. Ionizable compounds (acids and bases) are often separated by RPC with buffered mobile phases (to keep the analytes in a non-ionized state) or with ion-pairing reagents [10]

F. Selection of Detector and Wavelength

After the chromatographic separation, the analyte of interest is detected by using suitable detectors. Some commercial detectors used in LC are: ultraviolet (UV) detectors, fluorescence detectors, electrochemical detectors, refractive index (RI) detectors and mass spectrometry (MS) detectors. The choice of detector depends on the sample and the purpose of the analysis. In case of multicomponent analysis the absorption spectra may have been shifted to longer or shorter wavelengths compared to the parent compound. Therefore the UV spectra of target analyte and impurities must be taken and overlaid with each other, and the spectra should be normalized due to different amounts present in the mixture. A wavelength must be chosen such that adequate response is for most of the analytes can be obtained [19-20]

V. Sample Preparation

Sample preparation is a critical step of method development that the analyst must investigate. For example, the analyst should investigate if centrifugation (determining the optimal rpm and time) shaking and/or filtration of the sample is needed, especially if there are insoluble components in the sample. The objective is to

demonstrate that the sample filtration does not affect the analytical result due to adsorption and/or extraction of leachable. The effectiveness of the syringe filters is largely determined by their ability to remove contaminants/ insoluble components without leaching undesirable artifacts (i. e., extractable) into the filtrate. The sample preparation procedure should be adequately described in the respective analytical method that is applied to a real in-process sample or a dosage form for subsequent HPLC analysis. The analytical procedure must specify the manufacturer, type of filter, and pore size of the filter media. 12The purpose of sample preparation is to create a processed sample that leads to better analytical results compared with the initial sample. The prepared sample should be an aliquot relatively free of interferences that is compatible with the HPLC method and that will not damage the column [20-21]

VI. Method Optimization in HPLC

Most of the optimization of HPLC method development has been focused on the optimization of HPLC conditions [17] The mobile phase and stationary phase compositions need to be taken into account. Optimization of mobile phase parameters is always considered first as this is much easier and convenient than stationary phase optimization. To minimize the number of trial chromatograms involved, only the parameters that are likely to have a significant effect on selectivity in the optimization must be examined. Primary control variables in the optimization of liquid chromatography (LC) methods are the different components of the mobile phase determining acidity, solvent, gradient, flow rate, temperature, sample amounts, injection volume, and diluents solvent type. This is used to find the desired balance between resolution and analysis time after satisfactory selectivity has been achieved. The parameters involved include column dimensions, column-packing particle size and flow rate. These parameters may be changed without affecting capacity factor or selectivity [16]

VII. Conclusion

This review describes HPLC Technique used in food analysis. Knowledge of the physiochemical properties of the food is of utmost importance prior to the any HPLC method. The selection of Column, buffer, detector and wavelength and another conditions composition (organic and pH) plays a dramatic role on the separation selectivity. Final optimization can be performed by changing the gradient slope, temperature and flow rate as well as the type and concentration of mobile-phase modifiers. Optimized method is validated with various parameters (e.g. specificity, precision, accuracy, detection limit, linearity, etc.).

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