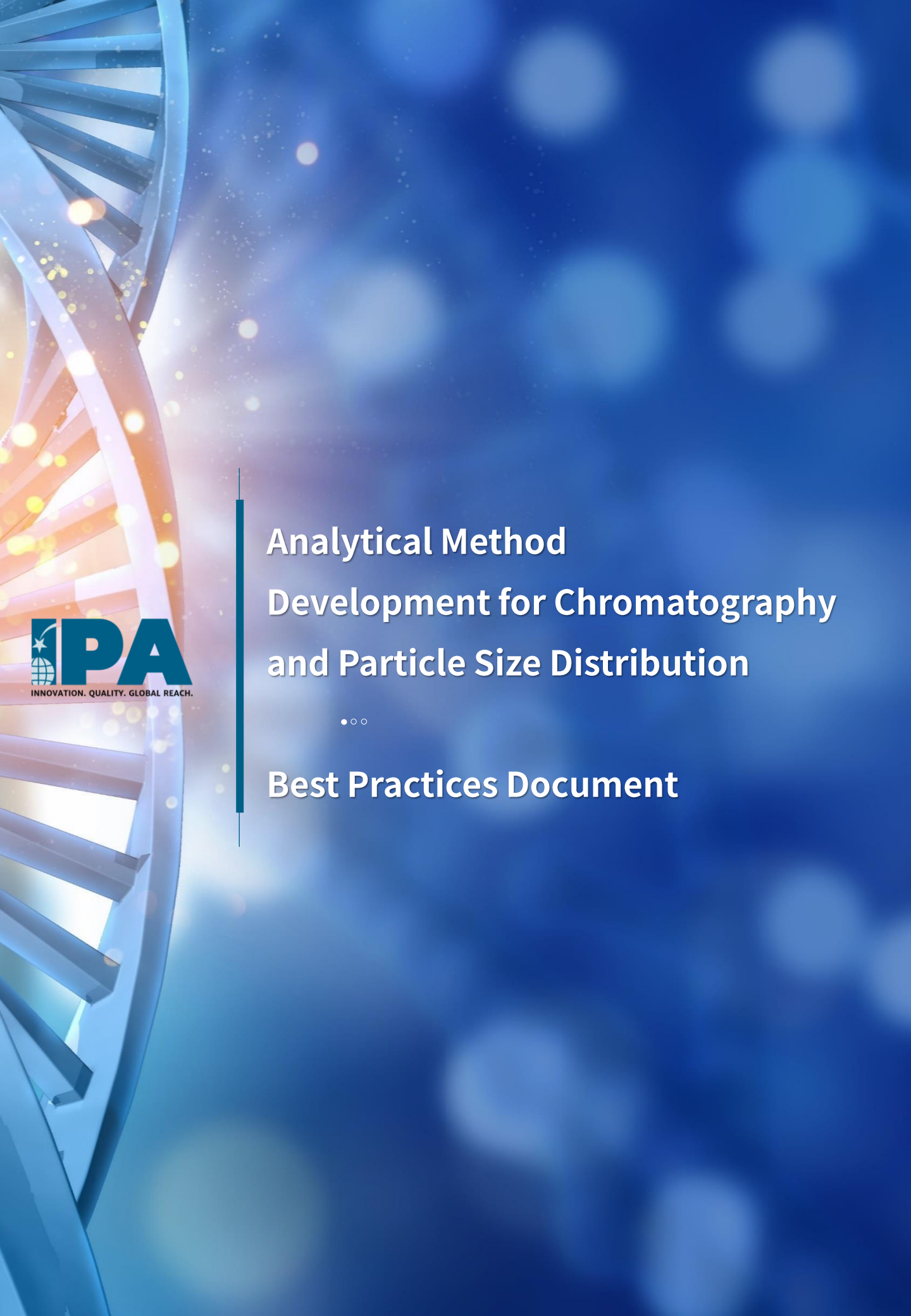

Analytical Method Development for Chromatography and Particle Size Distribution

Best Practices Document

CONTENTS

PREFACE

In April 2015, The IPA launched its Quality Forum (QF) to help Indian pharmaceutical manufacturers to achieve parity with global benchmarks in quality. The QF made a commitment to a multi-year journey to address key issues facing the industry and develop best practices.

The QF focused on several priority areas in the last four years, namely, Data Reliability, Best Practices & Metrics, Culture & Capability, Investigations, etc. It took upon itself the challenge of developing a comprehensive set of Best Practices Documents for several of these topics. In this document, we focus on best practices for Analytical Method Development for Chromatography and Particle Size Distribution. We had released a comprehensive set of Data Reliability Guideline in February 2017, Process Validation Guideline and Good Documentation Practice Guideline in February 2018, Investigation of non-conformities in February 2019 and Handling Market Complaints Best Practices in February 2020.

The five participating companies in the QF nominated senior managers to study the best practices and frame the guidelines. They are: Sutritho Mukopadhyay (Cadila Healthcare); B M Rao (Dr Reddy's); Pritesh Upadhyay (Lupin); Ashutosh Sharma (Sun); and Tripti Gandhi (Torrent). The IPA wishes to acknowledge their concerted effort over the last 12 months. They shared current practices, benchmarked these with the existing regulatory guidance from the USFDA and other regulatory bodies such as UKMHRA, WHO, etc., developed a robust draft document and got it vetted by a leading subject matter expert and regulatory agencies. The IPA acknowledges their hard work and commitment to quality.

The IPA also wishes to acknowledge the CEOs of six member-companies who have committed their personal time, human resources and provided funding for this initiative.

This document, to be released at the IPA's Advanced GMP Workshop 2020, will be hosted on the IPA website www.ipa-india.org to make it accessible to all manufacturers in India and abroad.

MUMBAI
OCTOBER 2020

Section 1 Chromatography Development

1 Purpose

To lay down the procedure of analytical method development for drug products, using Liquid and Gas Chromatography techniques.

2 Scope

- This Best Practices Document provides detailed information about the analytical method development to be carried out as per ICH guidelines.
- This Best Practices Document is applicable for the analytical method development for drug products. It is meant to drive step-by-step activities of method development. It is applicable for various test procedures like related substances, assay, content analysis, content uniformity, dissolution, residual solvents, etc.

3 Responsibility

- Research Associate/Associate Scientist/Research Scientist/Senior Research Scientist/ Designee is responsible for the analytical method development.
- Associate Scientist/Research Scientist/Senior Research Scientist/Principal Scientist/ Designee is responsible for reviewing the method development trials and the generated data.
- Senior Research Scientist/Principal Scientist/Designee is responsible for the approval of the analytical test procedure and of the analytical method.

4 Definition

A method development is a set of experimental conditions designed to perform accurate and precise analysis of a sample as per requirement.

5 Procedure

Method development scientists are expected to have good understanding of the drug product on which they are working, basic knowledge of analytical techniques and method validation, and transfer requirements of the respective tests and techniques. Some of the recommended pre-reads to actual method development are:

- USP <621> Chromatography.
- ICH Quality guidelines Q2 & Q3 (A-D).
- iii) In-house Analytical Method Validation SOP.
- iv) Patient information leaflet of the drug product and product specific guideline(s), if any.

I. Literature Search

- Literature search is important in order to collect basic information regarding the physico -chemical nature of the drug substance, impurities, and available analytical methods.
- Literature can be collected from in-house data, Pharmacopeia, Pharmacopeial Forum, Pharma Europa, libraries, Internet sites like Science-Direct, PubMed, DrugBank, Rx-List, and various other sources.
- An overview of Material Safety Data Sheet (MSDS) is mandatory since that contains information on the potential hazards (health, fire, reactivity and environmental) and how to work safely with the chemical product. It is an essential starting point for the development of a complete health and safety program.

II. Physico-Chemical Properties

The following properties should be considered:

- Chemical structure: presence of chromophoric groups, conjugation, auxochrome, ionic nature, molecular mass of compound, etc.
    - Physico-chemical properties of excipients: e.g., solubility, chromophoric nature, reactive impurities of excipients, acidity/basicity, swelling, floating properties, etc
    - pH, logP and pKa from literature or ACD software
    - Melting point, boiling point, vapor pressure
    - Sample/impurities solubility: solubility of analyte in different solvents
    - Sample stability and storage: hygroscopicity, photostability, thermal stability, etc
    - Polymorphism

III. Dosage Form Information

The following factors should be considered:

- Drug product description and number of strengths. In case of products with multiple strengths, details like dose proportionate (i.e., scale up/down) or pseudo dose proportionate (i.e., look-alike) should be taken into account. In case of pseudo dose proportionate, details of the nature of excipient(s)

compensated should be taken into account.

- ❖ Maximum daily dose and allowable impurities limit and genotoxic impurities limit.
- ❖ Packaging and storage information.
- ❖ List of excipients.
- ❖ List of solvents used in DP manufacturing process, API(s) and excipient(s).

IV. Analytical Target Profile (ATP)

- ❖ ATP is the summary of quality requirements of a method. An ideal profile should consider all requirements of a method. It should be classified into three categories (please refer to Table 1).

Table 1

Requirement	Parameters
Method perspective	❖ Method with adequate resolution between peaks of interest (resolution ≥ 1.5) ❖ With high detection sensitive (s/n ratio ≥ 10 at reporting threshold) ❖ Good peak shape to ensure accurate quantification (USP tailing < 2, theoretical plates >2000) ❖ Stability indicating power ❖ Chromatography with good baseline to ensure auto integration
Regulatory perspective	❖ Specificity ❖ Range (linear, precise, and accurate in the desired range) ❖ Other regulatory requirements (like %RSD for assay as per USP <621> and other requirements of ICHQ2 and USP recommendations).
Business perspective	❖ Simplified procedure, user and environmental friendly ❖ High throughput (less chromatographic run time and sample preparation time) ❖ Affordable (cost and durability of column, availability of equivalent column in market, cost of solvents, reagents etc) ❖ Preference of the method which can be executed with available resources (before demanding any special resources) ❖ Robust and rugged (performance of the method with respect to day-to-day/system/analyst variation and ability to tolerate small variations during execution) ❖ Preference of single method (like same column, mobile phase) for all the tests (like RS, assay, and dissolution).

V. Selection of Analytical Techniques:

- ❖ Selection of analytical techniques should be based on several considerations such as available literature and methods, physico-chemical properties, and available analytical resources.

- ❖ For non-chromophoric, volatile and thermally stable compounds GC is a preferred technique. However, for such compounds by converting to a stable, non-volatile, chemical derivative; other techniques (such as LC, UV spectrophotometry) should be attempted.

- ❖ For liquid chromatography, please refer to Chart 1 (given at the end) for further guidance.

Vi. Method Development by Liquid Chromatography

- This is applicable for related substances, assay, content analysis, content uniformity, dissolution, etc.
- The formulated sample would be received from various department(s)/location(s) like FRD, outsource parties, and manufacturing sites for method development.
- **Approach 1: Method development for drug product using available reference(s) (i.e., pharmacopeial reference, reported literature and/or any other references).**
- In case of the literature reference method, it is important to evaluate the suitability of the reported method for the drug product and make necessary changes if it does not meet the requirement of ATP.
- **Approach 2: Method development for the drug product in the absence of adequate literature and references.**
- In the absence of enough literature or in case the existing methods are not suitable for the dosage form, method development should be started from the fundamentals by considering physico-chemical properties of the analyte and ATP.

Method development and optimization should be initiated, preferably with the help of softwares like ACD, Design Expert, etc. Conventional approach can be adopted based on prior knowledge and experience.

VII. Column Selection:

For Reverse Phase Chromatography: The choice of the column (stationary phase) can be made based on the following parameters and their suitability for the current applications.

- Particle size of the silica
- Functional groups or polymeric layers that are attached to the silica surface; method development should preferably be initiated with C-18 or C-8 Columns
- Carbon loading
- Surface area
- Column length and internal diameter
- Details of end-capping and type of end-capping material
- Column-to-column variability
- Concern of pH range in which column packing material is capable of remaining unaffected

In general, a column can be used in the pH range of 2 to 8 of mobile phase. However, based on the requirement, special columns are available that can be used when the pH is outside the above range.

Table 2: Types of Column

Type of Column	Remark
C-18 ("octadecyl", "ODS")	Rugged; widely used; polar compound can be retained well when compared to a column with shorter carbon chain.
C-8 ("octyl")	Similar to C18. Polar compound can be retained well when compared to a column with shorter carbon chain.
C-3 and C-4	Less retentive; less stable; used mainly for peptides and proteins.
Phenyl and phenethyl	Moderately retentive; selectivity can be changed. Useful for the separation of compounds with closely related aromatic structure (use of pi-pi interaction for separation).
CN ("cyano")	Moderately retentive; normal phase.
NH2 ("amino")	Weakly retentive; more often used for normal phase; less stable.
Polystyrene	Stable for 1 < pH < 13; good peak shape and lifetime. Selectivity change often found less efficient.
HILIC (Hydrophilic Interaction Liquid Chromatography)	For the separation of polar or ionic compound. Use hydrophilic stationary phase with reverse-phase type eluent.

For Normal Phase Chromatography: the preferred options are silica stationary phase packing column, amino, diol, cyano or chiral stationary phase columns that are necessary for analysis.

A conventional column length is 50 mm to 250 mm for high resolution with 3 to 10µm particles.

VIII. Mobile Phase Selection:

I. **For Reverse Phase Chromatography:** The selection of mobile phase is a very important parameter in HPLC method development as the selectivity is altered by changing the mobile phase.

a. **Solvent Selection:** The following properties should be evaluated for selection of the organic solvent:

- Polarity
- Compatibility with the detection system
- Miscibility
- Solubility of the analyte in the selected solvent
- UV cutoff values of solvents.

- A good starting point is a mixture of water and a polar organic solvent (methanol/ acetonitrile, etc.).
- The effect of mobile phase polarity on elution time can be tested at a few different solvent proportions. If greater selectivity is required, a mobile phase comprising of multiple solvents may be used.
- Commonly used solvents are acetonitrile and methanol; limited use of tetrahydrofuran, isopropyl alcohol, etc. may be considered. As a general guide, the relative solvent polarity chart can be referred to for mobile phase selection.
- It is preferable to start with simple combinations of water/buffer, methanol and acetonitrile as binary or tertiary mixtures.
- Commonly used solvents (arranged on increasing polarity) and respective UV cutoff are given in Table 3

Table 3

Solvent	UV cutoff
1,1,2-Trichlorotrifluoroethane	231
Hexane	195
Cyclohexane	200
n-Butyl Chloride	284
Toluene	288
o-Xylene	288
Chlorobenzene	287
Ethyl Ether	215
Dichloromethane	233
n-Butyl Alcohol	215
Isopropyl Alcohol	205
Isobutyl Alcohol	220
n-Propyl Alcohol	210
Tetrahydrofuran	212
Chloroform	245
Methyl Isobutyl Ketone	334
Ethyl Acetate	256
Methyl Ethyl Ketone	329
1,4-Dioxane	215
Acetone	330
Methanol	205
Acetonitrile	190
N,N-Dimethylformamide	268
Dimethyl Acetamide	268
N-Methyl pyrrolidone	285
Dimethyl Sulfoxide	268
Water	190

b. Buffer Selection :

- Buffers are used in HPLC mobile phase preparations in order to achieve reproducible chromatography.
- Buffers are needed when the sample contains ionizable species or if the sample matrix is acidic or basic in nature.
- In reversed phase chromatography, samples are separated based on their hydrophobicity.
- A compound in its ionized form becomes more polar and less retained in RPLC. Similarly, a compound in its un-ionized form becomes less polar and hence more retained in RPLC. It is important to note that acid compounds are in ionized state as the pH increases, and basic compounds are in ionized state as the pH decreases. A graphical representation of this phenomenon is given in Figure 1.

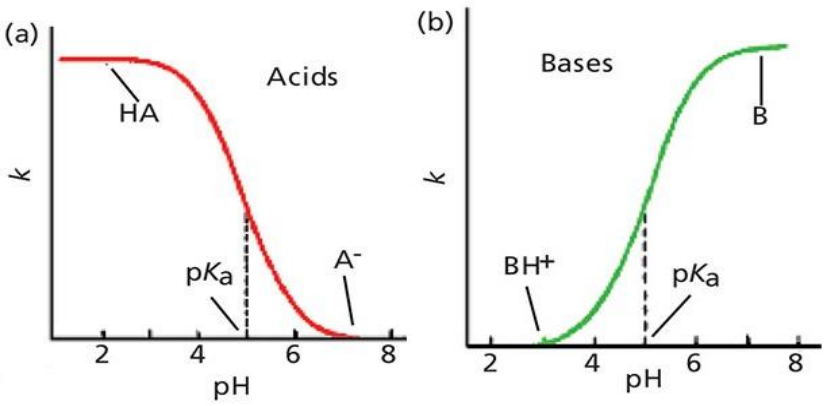

Figure 1: Retention behavior/factor (k) versus pH; (a): Acidic compounds (b): Basic compounds

- To develop a rugged method, buffers should be employed preferably at least 1 pH unit away from the analyte(s) pKa. This is derived from the Henderson-Hasselbach Equation:
- $pH = pKa + \log([A^-]/[HA])$
- Essentially, operating at a pH near the pKa of the sample analyte means that it will be in a partially dissociated state.
- The analyte will be partially in its weak acid or base form and partially in its conjugate form. This will cause peak distortion in the chromatography due to poor peak reproducibility.
- Operating with a mobile phase at least 1 pH unit away from the analyte pKa ensures that the sample would be in a single state almost entirely (i.e. either in ionized or unionized state).
- Once the desired pH range for the mobile phase is determined, choosing the right buffer can begin.
- Buffer capacity is optimized at or near a pH equal to the pKa of the buffer. As a rule of thumb, most buffers work suitably well within ± 1 pH unit of their pKa.

Table 4: Buffers for Reversed Phase

Buffer	pKa		Buffer Range	UV Cutoff (nm)
Phosphate	pK_1	2.1	1.1-3.1	210
	pK_2	7.2	6.2-8.2	
	pK_3	12.3	11.3-13.3	
Citrate	pK_1	3.1	2.1-4.1	230
	pK_2	4.7	3.7-5.7	
	pK_3	5.4	4.4-6.4	
Formate		3.8	2.8-4.8	210
Acetate		4.8	3.8-5.8	230
Tris (Hydroxymethyl) aminomethane		8.3	7.3-9.3	220
Borate		9.2	8.2-10.2	210
Triethyl amine		10.8	9.8-11.8	200

- Another consideration for a buffer selection is the type of detector being used. For example, citrate may not be suitable for low UV applications due to its high UV cutoff.
- In some cases, buffer selection should be based on experimental observation and prior knowledge.
- Likewise, if mass spectroscopy detection is used, a volatile buffer such as TFA, acetate or formate should be employed.
- Use of buffers/ion-pairing reagents/other modifiers (such as triethylamine, diethylamine, TFA, etc.) may be considered in the mobile phase to enhance the reproducibility, selectivity, or peak shape.
- When the use of a simple buffer does not deliver desired results, the mobile phase with ion-pair reagent can be attempted.
- Primarily, a buffer is used to regulate the pH and the acid-base equilibrium of the solute in the mobile phase. They may also be used to differ the retention times of ionizable compounds.
- The primary selection criteria of a buffer are:
    - Buffer capacity and its effective range
    - Miscibility of the buffer and its compatibility with the detector
    - Ability of the buffer to achieve the desired separation
- Use of volatile buffers and modifiers should be avoided for lower wavelength applications. In general, the retention of analytes can be modified by changing the pH. The pH modification should be done by considering the counterion part of the respective buffer.
- The most commonly used buffers and pKa values are summarized in Table 3 (above) for information.

II. For Normal Phase Chromatography:

- In order to ensure the solubility of the analyte, the first step is to check the compatibility of the analyte with the solvent.
- Initially, one should start with n-Hexane/n-Heptane in combination with ethanol or isopropyl alcohol.
- Selection of organic solvents and its ratio can be finalized during optimization of the method.
- It is important to refer to the column manuals before use and to ensure the compatibility of the solvent with the column.
- All the chemicals and reagents should be of high purity grade (AR grade, spectroscopic grade, etc.). Organic solvents should be of HPLC grade.
- Suitable modifiers (trifluoro acetic acid, diethyl amine and triethylamine) should be used in the mobile phase, if required. One should start with the minimum modifier concentration to prepare the mobile phase and progressively optimize the concentration.
- Degassed mobile phase should always be used without filtration.

a. Mode Of Elution:

- For Reverse Phase: Method development can be initiated by using isocratic or gradient elution based on requirement.
- It is usually recommended to mix a small portion of an aqueous medium (in organic portion) to avoid the pumping problem. Similarly, a small amount of organic solvent in aqueous phase should be added to minimize microbial growth.
- Care should be taken to avoid the precipitation of buffers in gradient mode of the mobile phase. It is also recommended to check the back pressure/physical observation at optimum proportion of mobile phase.
- In gradient method, it is important to ensure that no analyte is eluted at the re-equilibration phase.
- It is important to provide for adequate re-equilibration with initial composition at the end of gradient run. Also, it is important to ensure a consistent gradient pattern.

IX. Detector Selection:

Various detectors used in a HPLC instrument include UV-Visible detector, photodiode array detector, fluorescence detector, conductivity detector, refractive index detector, electrochemical detector, mass spectrometer detector, Corona detector (CAD-Charged aerosol detector) and evaporative light scattering detector. UV-Visible detectors are typical in many laboratories as they can detect a wide array of compounds.

UV detector is preferred when chromophoric groups are present in the compound and have adequate response for the detection.

Analytical derivatization is mainly used for following reasons:

- To permit analysis of compounds with inadequate volatility or stability, and/or
- To improve chromatographic behavior (like peak symmetry, retention, resolution), and/or
- To improve detectability

Examples for functional groups present in the molecule and suitable derivatizing agents are given in Table 5.

Table 5

Functional group present in the analyte	Derivatization reagents
Amine	Acyl chloride, aryl sulphonyl chloride, isocyanates, isothiocyanates, etc.
Carboxylic acid	Phenacyl bromide, methyl phthalimide, O-p-nitro benzyl-N,N-diisopropylisourea (p-NBDI), etc.
Hydroxy compounds	Acyl chloride (like benzoyl chloride derivatives), phenyl isocyanate, etc.
Carbonyl compounds	2,4-DNPH, p-Nitro benzylhydroxylamine, etc.

Derivatization can be done at the pre-column stage (prior to analysis) or at the post- column stage (after chromatographic elution).

I. UV and PDA detectors:

PDA detectors are widely used to assure spectral peak-homogeneity. It is mainly used during stress ddpplication, for UV spectral identification and wavelength selection.

One should select a wavelength at which the highest response of analyte/impurity should be obtained. However, in some cases, a wavelength can be chosen different to the one for highest response, in order to reduce the interference from diluent/blank or mobile phase, if any

Wavelength selection can also be done based on the optimum response of each component in the sample matrix. Dual or multi wavelength strategies can also be adopted to monitor one or two components in a mixture, if required.

II. ELSD detectors:

The evaporative light scattering detector is far more useful for on-line lipid quantification than the commonly used UV detector. It works by measuring the light scattered from the solid solute particles remaining after nebulization and evaporation of the mobile phase.

III. Corona Detector(CAD):

This type of detector is also very useful for non-chromophoric compounds. It operates by nebulizing the mobile phase from HPLC using a nitrogen gas stream.

IV. MALS detectors:

Multi-Angle Static Light Scattering (MALS) detector performs absolute characterization of the molar mass and size of macromolecules and nanoparticles in solution, offering superb sensitivity.

This detector measures the intensity of the scattered light simultaneously at three angles. The molar mass values can be measured with the TREOS range from 200 Da to 10 MDa (the upper mass limit is primarily determined by the radius limit, with a sensitivity as low as 0.4 µg/mL BSA (a 66.4 kDa macromolecule).

The measurable RMS radius ranges from 10 to 50 nm and can be extended up to 150 nm with specific conformation models over a wide range of molecular weight, size and concentration.

A MALS detector should be used to determine molar masses, RMS radii, polydispersities, branching ratios, etc. Certain calibration constants and system parameters must be determined and entered into ASTRA. Otherwise, any other available detector may be used as per its applicability and analyte property.

V. Fluorescence detectors:

Fluorometric detectors are sensitive to compounds that are inherently fluorescent or that can be converted into fluorescent derivatives.

VI. Refractive index detector:

These are used in cases in which chromophoric groups are absent in the compound. Since a refractive index detector cannot be used in gradient elution, ELSD (Evaporative Light Scattering Detector) can be applied.

X. Flow Rate:

Flow rate of the mobile phase should be adjusted for desired retention, separation of analytes, back pressure, and run-time.

XI. Column Oven Temperature:

Separation of analyte peaks can be enhanced by selecting the right column temperature depending on the nature of the components.

Higher column temperature reduces system back pressure by decreasing mobile phase viscosity, which in turn allows use of longer columns with higher separation efficiency.

The overall separation and peak symmetry can be improved by simultaneous changes in column temperature and mobile phase composition.

Usually, a column oven temperature within the range of 10°C to 50°C is preferred. If the column temperature is required to be more than 60°C, then the capability of the column packing material and physical properties of the same should be considered.

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V. Auto Sampler Temperature:

Auto sampler temperature should be maintained between 4°C and 25°C based on solution stability. If the solution is not stable at room temperature, then the auto sampler temperature should be kept at 10°C or lower. One must ensure that there is no recrystallization or precipitation of solutes when the solution is kept at cold temperature.

If the diluent is of fully organic solvent, there could be the possibility of evaporation of solvent from the vials (if these are not tightly crimped or are repeatedly punctured). This could make the concentration of the solution different from the actual preparation and may adversely affect the result of assay. A cooled auto sampler is to be preferred in such cases.

VI. Injection Volume:

The injection volume of the chromatographic method should be adjusted based on the response of the analyte and the sensitivity of the instrument. A good starting point is that the injection volume should be in the range of 5 to 20 µL for HPLC. However, this can be varied based on the specific application or prior experience. In order to improve the peak symmetry and resolution, injection volume should be kept low if the diluent composition is different from the mobile phase. It should be ensured that the injection volume of the method is within the calibration range of the system. If the desired injection volume of the method is beyond the calibration range of the injector, the injector should be calibrated for the desired injection volume.

XIV. Sample Preparation:

For sensitive molecules, the material of construction, grade and quality of volumetric flasks, pipettes, other measuring equipment and vials should be studied and defined in the test procedure. Material of construction should be glass (soda-lime, borosilicate, etc.) or plastic (polypropylene, polymethylpentene, etc.) and it should be transparent or low-actinic.

These are some important points to be considered while optimizing the sample preparation procedure:

- Sample matrix
- Sample concentration
- Diluent selection
- Extraction procedure
- Clarification procedure

I. Sample matrix:

These are some important points to be considered

- Physical nature of sample (solid, semisolid, solution, suspension, etc.)
- Drug to excipient ratio
- Stability of sample matrix (hygroscopicity, thermal, photo stability, etc.)
- Safety of API

Note: For low content/high potent drug, dropping of an intact tablet/capsule is preferred.

For high content drug, the tablet should be crushed, or the content of the capsule should be collected and the required amount should be taken.

Some coating material may interfere in the process of grinding and hence the uniformity of the drug could be affected.

II. Sample concentration :

The concentration of the test and standard should be set such that response measures in seven to eight-digit area (µAU*sec) for assay and RS may be achieved. Other concentrations are also acceptable based on chromatographic output.

Note: Sample concentration of RS test should be optimized to achieve the LOQ below the reporting threshold.

Sample concentration should be optimized in order to get the desired range in assay method.

III. Diluent selection :

Some important considerations are:

- Solution stability.
- Solubility/miscibility for sample (these should be set preferably at twice the desired concentration).
- Peak shape and resolution.
- Aqueous: organic ratio and pH of diluent.
- Mobile phase compatibility.

Note: Mobile phase is the preferred diluent for better chromatography. If the diluent is of fully organic solvent, there could be the possibility of evaporation of the solvent from the vials (if these are not tightly crimped or are repeatedly punctured). This could make the concentration of the solution different from the actual preparation and may adversely affect the result of assay. A cooled auto sampler is to be preferred in such cases.

IV. Extraction procedure:

A typical sample extraction procedure includes sonication, homogenization, stirring, heating/cooling, vortex mixing, wrist action mechanical shaker, partition, Solid Phase Extraction (SPE), etc.

Note: The sonication time and temperature should be optimized. The frequency of the sonicator used should be noted. Some sonicators may not produce uniform frequency throughout its surface. Hence, it is important to study the sonication procedure by overcrowding of flasks in the sonicator bath or keeping the flasks on various places of the bath.

Requirements such as intermittent shaking/temperature/duration of extraction procedure should be studied thoroughly and defined in the test method.

In case of sample preparation by stirring, aspects such as stirring speed, time, temperature, and dimension of the magnetic bar should be defined.

Multiple extraction procedures may be adopted for preparation of the sample in order to obtain the desired accuracy.

Multiple sample extraction procedures should be employed. For example, extraction by sonication procedure should be critically compared with stirring and/or with a wrist action mechanical shaker.

Robustness study should be done for the critical steps of sample preparation procedures, with different makes of equipment used for sample preparation.

V. Sample clarification procedure :

Sample solution for injection into a LC system should be free of undissolved particles. In the drug product, there could be insoluble excipients and those should be removed by suitable means. Centrifugation, filtration, or a combination of both is the commonly employed technique to make the sample solution suitable for LC injection. Occasionally Solid Phase Extraction (SPE) and liquid-liquid partition are also used in sample clarification procedure.

In case of centrifugation, it is important to optimize the RPM (rotation per minute) and the time of centrifugation. If necessary, temperature during centrifugation should be studied and recommended.

Syringe filters are widely used during clarification procedures. The ideal qualities of a filter are:

- No adsorption or degradation of the compound of interest
- No leaching from the filters; this can be ensured by the absence of any extraneous peak in the chromatographic condition
- Mechanical stability to withstand the filtration of high particulate suspension.

Some of the commonly employed filter material and their characteristics are given in Table 6.

Table 6

Membrane material	Characteristic
Nylon	Compatible with many HPLC solvents (THF, alcohols, acetonitrile) but not with strong acids.
PVDF (Polyvinylidene difluoride)	Hydrophilic material for use with alcohols, hydrocarbons, biomolecules, ether, and acetonitrile.
PTFE (Polytetrafluoroethylene)	Chemically resistant to almost all solvents, strong acids and bases. Hydrophobic membrane should be pre-wetted when used with aqueous solutions.
PP (Polypropylene)	Hydrophilic material with resistance to most acids, bases, DMF, DMSO and alcohols. Not recommended for use with hydrocarbons, esters, or solvents such as acetonitrile.
PS/PES Polysulfone/ Polyethersulfone Variants	Commonly used with tissue culture and ion chromatography samples. Stable with many strong bases and alcohols, but few HPLC solvents (as it is hydrophilic). Not compatible with acetonitrile.
CA (Cellulose acetate)	Use with aqueous solutions and a few hydrocarbons only. Low protein binding, hence good for many biological samples. Not compatible with acetonitrile or DMSO.

Depending on the sample volume, the diameter of the filters should be chosen. Based on the application, the pore size should be chosen. The filters should be saturated with the diluent prior to the collection of the actual sample solution. For this purpose, the initial few mL of the filtered solution should be discarded, and the volume of the initial discarding should be defined.

VI. Forced Degradation Studies:

Physical degradation (thermal, photolytic and humidity) and chemical degradation (acid, alkali and oxidative) studies of the drug product should be conducted. Degradation of drug substances between 5% and 20% has been accepted as reasonable for validation of chromatographic assays. The degradation due to any one stress condition should conform to mass balance.

Placebos of the respective samples should be exposed to same degradation condition.

During FD study the treated sample pH should be verified against the control sample solution pH.

While performing forced degradation studies, initially diluent and reagent quantity of each should not exceed 10% of total volume of flask.

Based on available information or experience of the drug product, the degradation should be studied under mild or harsh condition. The drug products which are very susceptible to degradation should be studied under mild conditions. Comparison of degradation products under mild and harsh conditions may give information about secondary degradation.

Under stress conditions, the temperature may be limited as per the composition of the organic solvent in the diluent.

Table 7 : Maximum stress condition

Type of study	Condition and Duration
Acid degradation	0.1 to 5 N HCl at RT to 80°C, few hours to 15 days.
Alkali degradation	0.1 to 5 N NaOH at RT to 80°C, few hours to 15 days.
Oxidative degradation	a) Exposure 0.3 to 30% H2O2 at room temperature up to 15 days. A slight high temperature (40° C) shall be applied. b) 5 mM Fe III or Cu II solution at RT.
Photo degradation	Fluorescent & UV light (ICH Q1B) 1.2 million lux hours, 1 to 4 cycles.
Thermal degradation	Up to 70°C for 21 days.
Thermal humidity	Up to 60°C/75% RH for 21 days.

Note: Potassium permanganate solution should be used as an alternative oxidation reagent.

The degradation impurities should be confirmed by hyphenated techniques like LCMS, GCMS, etc.

Since it is preferable to achieve about 5 to 20% degradation, suitable exposure conditions should be applied based on the nature and characteristic of the molecule.

No further justification is needed if degradation is achieved to about 5 to 20% in all or any one of the degradation conditions.

Based on the above studies, if no degradation is achieved, a scientific justification for the same should be provided.

If mass balance is not achieved in the range of 95% to 105%, it should be investigated and scientifically justified.

Some of the reasons for getting mass balance <95% are:

- Degradation impurity could not have eluted in the method
- Degradation impurity could have eluted at void volume and neglected as chromatographic disturbance
- Degradation impurity might have merged with degradation reagent(s) like hydrogen peroxide and/or elution of degradation impurity under counterions of the drug like tartrate, fumarate, maleate, etc., and/or elution of degradant peak at the RT of active peak and/or elution at the RT of placebo peak
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- Degradation impurity might be volatile
- The drug is not completely extracted from the degraded sample. Some of the reasons are incomplete neutralization of acid/base, trapping of drug in degraded placebo matrix, etc
- Peak of degradation impurity would be distorted and hence might be underestimated.
- Degradation impurity might be insoluble.
- Volatile/sublimation nature of the drug as such or when it loses its counterion.
- Due to water gain in physical degradation.

Some of the reasons for getting mass balance >105% are:

- Degradation impurity formed could have higher response. For example, the unknown impurity formed during degradation could have a different lambda max than the wavelength of the method.
- Co-elution of degradation impurity and/or placebo degradation at the RT of active/impurity peak.
- Degradation of counterion. For example, degradation of maleic acid to fumaric acid is obvious in acid and thermal degradation condition. Appearance of fumaric acid may be calculated as an impurity whereas the assay of drug remains unaffected.
- External contamination
- Due to water loss in physical degradation.

The developed method should be capable of known impurities and drug substances against the degradants.

The homogeneity of known peak should be confirmed by peak purity tool in PDA.

The degradants that are detected in the stress applications can provide information about the impurities that may be arise during the stability study.

Chemical degradation study is not applicable for normal phase chromatography.

Care must be taken to ensure that no degradant peak (formed during FD studies) is left un-eluted in the method. This should be assured by one of two methods: (i) in the isocratic method, the run time is increased, or a gradient run is applied at the end of the desired run; or (ii) in the gradient method, the time is extended or the organic ratio in the last phase of gradient program is increased.

XVI. Calculation Techniques :

- Response factor is the measurement of responses of equal concentrations of analytes

$$\text{Response factor} = \frac{\text{Slope or response of drug substance}}{\text{Slope or response of individual impurity}}$$

  Response factor should be used for calculation of impurities in the test for related substances by quantification against another peak.

- External standard method is widely used. Care must be taken to ensure that the standard concentration is close to the expected sample concentration. If the standard concentration is different from the sample concentration it should be justified adequately.
- Internal standard method is preferred when loss of sample is expected during the preparation or injection. The internal standard is a compound that is very similar, but not identical, to the chemical species of interest in the samples. Known quantity of internal standard is to be added to sample, standard, blank and placebo solutions. Ratio of the analyte signal to internal standard signal is used for calculation.
- Standard addition method is preferred in a situation where the sample matrix also contributes to the analytical signal (matrix effect). A typical procedure involves adding known quantity of standard to a fixed quantity of sample. In this method, sample and sample spiked with standard are analyzed. From the increase in analyte response in spiked sample, the original concentration can be computed by extrapolation.
- Diluted standard method is a preferred calculation technique for precise quantification of impurities in the sample solution. The diluted standard is prepared with respect to the maximum impurity level of the final test concentration (e.g., if test concentration is 1000 µg/mL and maximum impurity is 1.0 %, then the diluted standard would be 10 µg/mL).
- Normalization method is used for calculation of percentage of analyte in presence of other analytes that are expected to be present in the sample matrix. In the normalization method, sum of all the peaks appeared in the chromatogram is assumed to be 100%.

XVII. Execution, Interpretation and Conclusion of a Method:

- Every trial should have a clear objective, i.e. what is to be changed (e.g., mobile phase pH, column temperature, gradient program, etc.) in a trial and what is expected out of it.
- At the end of every trial, it should be thoroughly interpreted to understand its merits and demerits. Poorly interpreted data from a trial may lead to rejecting a good method.
- Enough saturation of the column should be given with mobile phase before injecting samples in each trial.
- Long time saturation is required for some ion-pair mobile phase and some impurities may behave erratically if the saturation is inadequate.
- If PDA spectra and response of impurities are similar, it would be difficult to track the elution order of impurities. If solutions mixed with impurities are used for development, it is recommended to use carefully designed solutions which contain each impurity at different levels during development trials (for example, impurity1 at 0.2%, impurity2 at 0.4%, impurity3 at 0.6% and API-100%).

- Any impurity which are known/designated as a process impurity, if, increased during FD studies or stability studies; there could be possibility of merging of a degradation impurity at the RT of process impurity. It is necessary to investigate through mass identification and if required, make necessary changes to separate the impurity.
- The performance of the method in a brand-new column and a well-used column of the same brand should be checked by noting down the number of injections made in the column. The method performance in different lots of column should also be checked and noted. Wherever possible, the performance of an alternative brand of column should be evaluated and noted.
- If the actual method is not compatible with a mass spectrophotometer, then an equivalent mass compatible method should be developed, by using volatile buffers, for the purpose of identification of unknown peaks as well as peak purity by LC-MS.
- Verification of the analytical method should be performed before validation of the method. The parameters to be measured and acceptance criteria should be in line with the current version of the SOP for validation of analytical methods.
- Statistical data can be generated from the method verification/method validation data as per the USP chapter 1210 (Statistical Tools for Procedure Validation) and as per the relevant requirement.

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XVIII. Method Development by Gas Chromatography :

(For residual solvents, related substances, assay, content analysis, content uniformity, dissolution, etc.)

The formulated sample will be received from various department(s)/location(s) like FRD, outsource parties, and other location(s) of the Company for method development.

Approach 1: Method development for drug product using available reference(s), (i.e. pharmacopeial reference, reported literature and/or any other references).

In case of published reference methods, the suitability of the reported method for the drug product should be evaluated and necessary changes should be made if it does not meet the requirement of ATP.

Approach 2: Method development for the drug product in the absence of adequate literature and references.

In the absence of enough literature or if existing methods are not suitable for the dosage form, then method development should be started from fundamentals by considering physico-chemical properties of the analyte and ATP. A conventional approach can be adopted based on prior knowledge and experience.

I. Carrier Gas Selection :

The carrier gas (mobile phase) for GC must be an inert gas that does not react with the sample components. Carrier gas is required to transport the vaporized solute molecules through the column during the partitioning process.

Carrier gases are compressible gases that expand with increasing temperature. This results in a change in the gas viscosity. The selection and linear velocity of the carrier gas shall affect resolution and retention times. Nitrogen, helium and hydrogen are the most commonly used carrier gases for capillary gas chromatographic analyses. Nitrogen and helium gases provide good efficiency. Hydrogen is combustible and is highly diffusive in air, hence one must be careful in its use.

II. Column selection :

Selecting the right column is a key step in GC method development. With the objectives as listed in ATP and by considering the sample matrix, the right column can be selected. Table 8 describes some of the important aspects of each column.

Table 8

Column Parameter	Commonly used Capillary columns (examples only, not limited to descriptions below)	Selection guide
Stationary phase	Non-polar like 100% polymethyl siloxane. Mid-polar like 5% phenyl polysiloxane. Strong polar like Polyethylene glycol (wax).	Depends on polarity of analyte select the stationary phase of the column. For non-polar compounds, non-polar columns are preferred.
Column length	Available in the range of 5 m to 100 m. Commonly used are 30 m and 60 m.	Better resolution can be obtained in a longer column. When the sample matrix contains few analytes and resolution is not a challenge, shorter column should be preferred.
Internal diameter (ID)	Available in the range of 0.1 mm to 0.53 mm.	Column ID has impact on efficiency (number of theoretical plates) and capacity (sample load). Narrower the ID, sharper is the peak and better the resolution. Sample capacity increases as column ID increases.
Film thickness	Available in the range of 0.1µm to 2 µm film thickness	Decreasing film thickness results in sharper peak and shorter retention.

Effects of phase film thickness are interdependent with column ID. The phase ratio (β), expresses the ratio of the gas volume and the stationary phase volume in a column:

β = column radius (µm) / 2 x film thickness (µm)

As a general rule, columns by β values should be selected as follows:

β Value <100 used for analysis of highly volatile and low molecular weight compounds.

β Value >400 used for high molecular weight compounds and trace analyses.

β Value = 100–400 used for general purpose analyses across a wide range of compounds.

β values are also useful when changing column ID and film thickness combinations for a particular analysis, because columns with the same phase ratio will provide very similar retention times and elution order under the same analytical conditions.

The most popular stationary phases used for gas chromatography are presented in Table 9

Table 9

Stationary phase	United States stationary phase Pharmacopoeia phase name	Commercial names	Additional comments
6%-Cyanopropylphenyl-Equivalent to DB-624	Equivalent to USP phase G43	DB-624, HP-624, Rtx- 624, CP-624, ZB-624	low/mid polarity
6% Cyanopropyl-phenyl-methylpolysiloxane	Equivalent to USP phase G43	DB-1301, HP-1301, Rtx- 1301, CP-1301, SPB-1301,	low/mid polarity
Polyethylene glycol (PEG)	Equivalent to USP phase G16	DB n WAX, HP n WAX, UPELCOWAX 10, CP-WAX 52CB, SUPEROX II, CB-WAX, Stabilwax, 007-CW, Carbowax, HP-Innowax, Rtx-WAX, ZB-WAX	high polarity
5% Phenyl-methylpolysiloxane	Equivalent to USP phase 27	DB-5, HP-5, Ultra-5, Rtx-5, CP-Sil 8 CB, SE-30, AT-5, ZB-5	Non-polar
Polydimethylsiloxane	Equivalent to USP phase G2	DB-1, Ultra-1, SPB-1, CP-Sil 5CB, Rtx-1, BP-1, ZB-1, AT-1	Non-polar
50% Trifluoropropyl-methylpolysiloxane	Equivalent to USP phase G6	Rtx-200, HP n 210, DB-210, VB -210	Mid/high polar

The column suitability for analysis is determined as below:

- A stationary phase with similar 'polar' characteristics as the analytes should be used to affect retention.
- Polar analytes require polar stationary phases to be retained in the stationary phase.
- Non-polar analytes require less/non-polar stationary phases.

III. Diluent selection and Extraction Procedure Optimization:

- Selection of diluent for the test and standard preparation are based on solubility data, chemical interaction of analytes and mode of injection (i.e., head space or liquid injection).
- The extraction procedure can be improved by increasing sonication time, intermittent shaking, homogenizing, stirring and vortex applications. Some sample gets soluble when it is subjected to head space incubation. For head space injection, complete solubility is not necessary, if acceptable accuracy and precision are achieved.
- For GC headspace application, commonly used solvents are dimethyl sulfoxide, N,N-dimethylformamide, N,N-dimethylacetamide, N-methyl pyrrolidinone, benzyl alcohol, water, etc. Other solvents/diluents can also be tried based on applicability.

- For GC liquid injection application commonly used solvents are methanol, acetone dichloromethane, cyclohexane, n-hexane, n-heptane, etc. Other solvents/diluents can also be tried based on applicability.
- Please refer to Table 10 for the boiling points of various solvents.

Table 10

Name of solvent	Boiling point (°C)	Name of solvent	Boiling point (°C)
Acetic Acid	118.0	Ethyl Acetate	77.1
Acetic Acid Anhydride	139.0	Ethyl Ether	34.6
Acetone	56.3	Ethylene Dichloride	83.5
Acetonitrile	81.6	Ethylene Glycol	197.5
Benzene	80.1	Heptane	98.4
Benzyl alcohol	205.0	n-Hexane	68.7
iso-Butanol	107.7	Hydrochloric Acid	84.8
n-Butanol	117.7	Methanol	64.7
tert-Butanol	82.5	Methylene Chloride	39.8
Carbon Tetrachloride	76.5	N-Methyl-2-pyrrolidone	202
Chlorobenzene	131.7	MTBE	55.2
Chloroform	61.2	Pentane	36.1
Cyclohexane	80.7	Petroleum Ether	35.0-60.0
Cyclopentane	49.3	iso-Propanol	82.3
Dimethyl Imidazole	204	n-Propanol	97.2
Dichloromethane	39.8	Pyridine	115.3
Diethyl Ether	34.6	Tetrahydrofuran	66.0
Dimethyl Acetamide	166.1	Toluene	110.6
Dimethyl Formamide	153.0	Trifluoroacetic Acid	71.8
Dimethyl Sulfoxide	189.0	Water	100.0
Dioxane	101.0	Xylene	140.0
Ethanol	78.3		

- Use of chemical/modifiers (such as water, sodium hydroxide/hydrochloric acid/ anhydrous sodium sulfate, etc.) in the diluents enhances the reproducibility/sensitivity and accuracy of result, and may be applicable based on specific requirement.
- It is important to ensure the solubility/miscibility and recovery of analyte and impurities in sample and standard. The compatibility of the solvent with the solvent/sample should be checked.
- Solvent interference shall be considered like common impurities present in GC solvents.
- Please refer to Table 11 for common impurities in GC solvents.

Table 11

Solvent	Common impurities
Acetone	Mesityl oxide, benzene, diacetone alcohol
Benzyl alcohol	Toluene
Dimethyl sulfoxide (DMSO)	Dimethyl sulfide (DMS)
Isopropyl alcohol	Acetone
Ethanol	Methanol, acetaldehyde
Methanol	Formaldehyde

IV. Column Oven temperature:

Column oven temperature impacts resolution, retention and peak shape of analyte. Column oven can be with isothermal condition or with temperature program.

a. Isothermal conditions are suitable for the analyses of single components or when resolution between analytes is not critical. The set temperature of the oven should be capable of eluting analyte(s) and diluent as well.

b. Column oven temperature gradient program is used where the last eluting compound (for example, diluent peak like DMSO) needs more time for elution and gives a broad peak, or where there is a separation problem in the area of the low boiling compounds.

Initial column oven temperature should be kept at 40°C or as low as 35°C to 10°C lower than maximum permitted temperature of the column. Generally, increase in column oven temperature leads to quick elution.

The column should be properly conditioned prior to analysis. Holding the column at 10°C lower than maximum permitted temperature of the column is required for 2-3 hours to condition the column. If stable base line is not obtained, the column conditioning should be extended overnight.

V. Carrier Gas and Flow Rate: :

- Diffusivity, viscosity, cost, safety, and its reactivity with analyte of a carrier gas determine its application. Diffusivity provides a measurement for the diffusion speed of a solute vapor in each gas. For helium and hydrogen, diffusivities are similar, but that of nitrogen is about four times lower. The diffusion speed of the solute in the carrier gas determines the speed of chromatography. Helium and nitrogen are almost of same viscosity whereas hydrogen is nearly half. Higher the viscosity of the gas, higher the back pressure.
- Flow rate of the carrier gas is adjusted in accordance with desired retention, separation of analytes, optimized linear velocity and run-time.
- Generally, flow rate can be set in linear velocity (cm/s), pressure (psi) and volume (mL/min) mode. Please refer to Table 12 for optimal flows/linear velocities vs. capillary diameter.

Table 12

Optimum range, linear velocity, and flow (40°C)						
Column ID (mm)	Nitrogen		Helium		Hydrogen	
	cm/s	mL/min	cm/s	mL/min	cm/s	mL/min
0.10	13-17	0.1-0.15	30-35	0.3-0.34	48-60	0.4-0.6
0.18	12-15	0.2-0.3	27-32	0.6-0.8	44-54	1.0-1.3
0.25	10-13	0.3-0.44	25-30	1.0-1.3	40-50	1.5-2.0
0.32	8-11	0.4-0.55	22-27	1.2-1.6	37-47	2.0-2.7
0.53	6-10	0.7-1.3	20-24	2.6-3.0	35-45	4.7-6.1

VI. Sample Injection:

- The injector temperature should be set to be just hot enough to assure "instantaneous" evaporation of the entire sample. For many samples, 250°-275° C is adequate. From this initial setting, higher or lower temperature settings can be tested in 20°C increments, while inspecting for any negative changes in peak width, peak area, peak shape or differences in repeatability. Setting too high an inlet temperature may lead to thermal degradation of analyte or back flash of solvent.

a. Liquid Injection:

- The injection volume of the chromatographic method is adjusted based on the response of analyte and the sensitivity of the instrument. Generally, injection volume should be selected as 0.2µl to 0.5µl for neat sample (liquid) injection and 0.5µl to 5.0µl for diluted (solid and liquid) sample and it may vary based on the specific application. For low injection volume, the sensitivity of the method should be checked, and for high injection volume, the peak shape should be verified at higher concentration levels.

b. Head space injection:

- Injection volume/time for headspace application should be selected as 0.05 min to 0.4 min vapor phase; this may vary for specific applications. This parameter should be optimized by instruments of different makes like Agilent/ PerkinElmer/Shimadzu, etc., based on their injection techniques. For loop injection, headspace sampler injection time can be optimized based on application.
- Temperature and duration of incubation in the headspace oven is optimized based on the boiling point of the analyte, its thermal stability and chromatographic response.
- The needle temperature is kept equal or higher than the headspace oven temperature and the transfer line temperature should be kept higher than that of the needle temperature; however, this is not a definite requirement.

VII. Injection Mode:

- There are two techniques used for transferring the vaporized sample onto the head of the column – split and splitless modes.

- In the split mode only a fraction of the vaporized sample is transferred on to the head of the column while the remaining is removed from the injection port via the split vent line.
- For trace analysis, the split mode may not be suitable; in this case the splitless mode is preferred.

VIII. Detection:

- FID (Flame Ionization Detector) and mass detector are widely used detectors in pharmaceutical analysis.
- FID can detect almost all organic compounds. Higher the amount of carbon present in the analyte, higher the expected response with FID. Mass detectors are used for quantification as well as identification of peak.
- FID detector requires hydrogen, air and make up gas. Generally recommended flow
- rate of these gases are: hydrogen in the range of 24 to 60 mL/min (optimum 40 mL/min), air in the range of 200 to 600 mL/min (optimum 450 mL/min), and make up gas 10 to 60 mL/min (optimum 50 mL/min). Nitrogen is a recommended makeup gas and helium is a good alternative. The hydrogen-to-air ratio should be between 8% and 12% to keep the flame lit.
- Please refer to Table 13 for common gas chromatography detectors with their characteristics.

Table 13

Detector Type	Characteristic
Flame ionization detector (FID)	Universal (organic/carbon compound)
Electron capture detector (ECD)	Selective (halogens and other electron withdrawing groups)
Photoionization detector (PID)	Universal (based on ionization energy)
Thermal conductivity detector (TCD)	Universal
Mass spectrometer (MS)	Universal

IX. Sample preparation:

- For liquid injection, the analyte and sample matrix should be completely soluble in the diluent.
- If the sample matrix is insoluble in any diluent, the analyte should be separated from the sample matrix by a suitable means (like extraction, centrifugation, filtration, etc.) and sent for analysis.
- For head space injection, the complete solubility of sample matrix is not necessary.
- The diluent for head space injection should be selected based on its ability to withstand the head space incubation temperature and duration.
- Optimized sample concentration should give acceptable LOQ (1/3rd or up to 50% of specification limit), linearity (high correlation coefficient), reproducible (less % RSD) and accurate (high % recovery).
- Content of highly volatile compounds (like dichloromethane, methanol, etc.) may decrease over time. Hence it is important to understand the nature of the analyte before troubleshooting the method.
- Some sample may contain more moisture, whereas the standard may have little or no moisture. The difference in moisture content in the sample and standard solution may have high impact in the analytical result and recovery.
- Derivatization procedure should be applied if the sample is non-volatile or thermally unstable or to improve the chromatographic behavior.
- Please refer to Table 14 for typical derivatizing reagent used in GC.

Table 14

Class of Reagent	Typical Reagent Compounds	Compounds Derivatized
Trialkyl silane	TMCS, HMDS, MSTA, MSTFA, BSTFA	Alcohols, phenols, carboxylic acids, thiols, amines, amides.
Haloalkyl acryl	Perfluorocarbonacyl (e.g. trifluoroacetyl-, pentafluoropropionyl-, heptafluorobutryl-).	Amines, phenols.
Haloalkylacryl	Alcohols, Reaction performed with excess of alcohol with acid catalyst (e.g., HCl, acetyl chloride, boron trifluoride).	Carboxylic acids.
Alkylation	Alkyl halides, diazoalkanes, acetals, N,N'-dimethylformamide dialkyl acetals.	Active hydrogen-containing compounds (e.g. -COOH, -SO2OH, -OH, -SH, -NH2, =NH)
Pentafluoro (PF) Phenyl	PF-benzoic anhydride, PF-benzyl bromide, PF-benzoyl chloride and others.	Alcohols, amines, phenols, carboxylic acids, ketones.
Oximes	Hydroxylamine or derivatives (Me, Et, or benzyl).	Ketones, aldehydes.

Codes:

TMCMS: Trimethylchlorosilane

HMDS: Hexamethyldisilazane

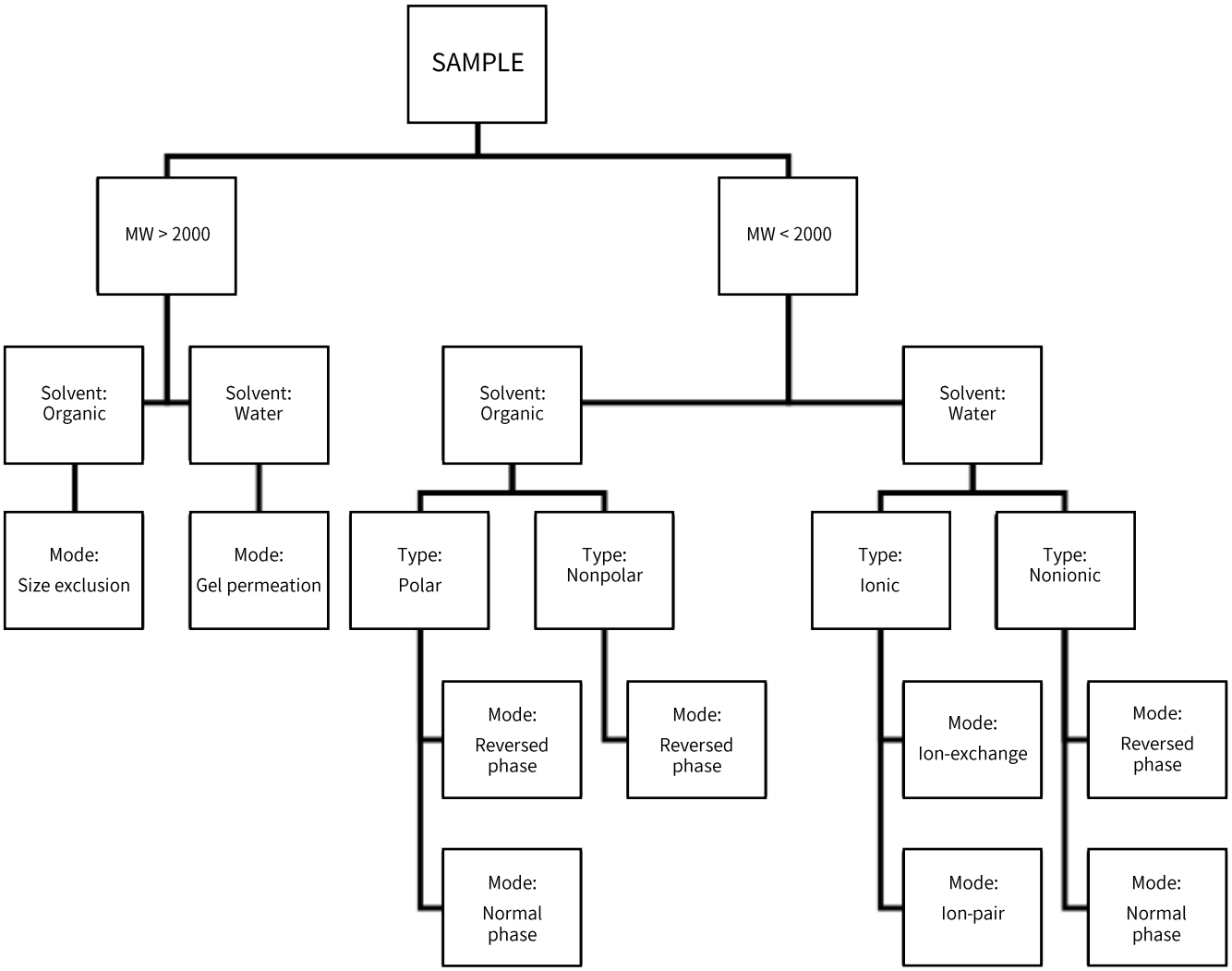
MSTFA: N-methyl-N-(trimethylsilyl) acetamide

MTBSTFA: N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide

XIX. Other consideration :

- For calculation techniques, forced degradation studies, execution, interpretation and conclusion of method, please read the section in liquid chromatography and consider the relevant points.
- Blank and placebo peaks in sample should be disregarded. If there are any extraneous peaks, other than blank and placebo, with S/N ratio greater than 10, these should be investigated.

Chart 1 : Flow diagram of HPLC Mode Selection (for Information)



6 Abbreviations

No.	:	Number
API	:	Active pharmaceutical ingredient
HPLC	:	High performance liquid chromatography
UV	:	Ultraviolet
M	:	Molar
%	:	Percentage
PDA	:	Photodiode array
ELSD	:	Evaporative light scattering detector
MALS	:	Multi-angle static light scattering detector
nm	:	Nanometer
mL	:	Milli Liter
min	:	Minute
°C	:	Degree centigrade
µl	:	Microliter
LOD	:	Limit of detection
LOQ	:	Limit of quantitation
N	:	Normality
ID	:	Internal diameter
RS	:	Related substances
µv	:	Micro volts
Au	:	Absorbance unit

7 References

- Practical HPLC Method Development, Second Edition. By Lloyd R. Snyder Joseph J. Kirkland, Joseph L. Glajch (John Wiley & Sons, Inc.).
- Modern Derivatization Methods for Separation Sciences. Edited by Toshimasa Toyo'oka (Wiley).
- Sample Preparation of Pharmaceutical Dosage Forms. Edited by Beverly Nickerson (aaps press, Springer)

Section 2 Particle Size Distribution

1 Purpose

To lay down the procedure for analytical method development of particle size distribution by Laser Diffraction.

2 Scope

This Best Practices Document provides a broad outline to the method development approach of particle size distribution using laser diffraction technique.

3 Responsibility

- Research Associate/Associate Scientist/Research Scientist/Senior Research Scientist/ Designee is responsible for the analytical method development.
- Associate Scientist/Research Scientist/Senior Research Scientist/Principal Scientist/ Designee is responsible for reviewing the method development trials and the generated data.
- Senior Research Scientist/Principal Scientist/Designee is responsible for the approval of the analytical test procedure and of the analytical method.

4 Definition

A method development is a set of experimental conditions designed to perform accurate and precise analysis of a sample as per requirement.

5 Procedure

I. Equipment Required:

- Laser diffraction particle size analyzer with relevant accessory (for example, dry or wet).
- Microscope.
- Vortex Mixture (Cyclo mixer).
- Mechanical shaker.
- Ultra sonicator (preferably of a reputed make with certified sonication power, typically with a frequency of around 30-40 MHz).
- Balance (1mg least count).
- Glass rod with round bottom on one side

II. Precautions to be taken during Measurement:

I. Importance of a Good Background:

- A good background signal in PSD measurement is as important as any other technique that generates data based on electrical signal.
- In an ideal case, the first detector should have a signal level of less than 100 and the signal should gradually reduce as the detector number increases
- Primary importance should be given to the cleaning of the equipment before and in between runs so that the background noise is kept as low as possible. This will have direct impact on the final data

II. Importance of Weighted Residual:

- Weighted residual is an approximate measure of the quality of the data produced
- A value of less than 1% is an expected one; for micronized particles (less than 10 µ) less than 2% is satisfactory
- Higher values may be possible; however, these need to be investigated and confirmed, since these may be background noise which may lead to a higher weighted residual leading to selection of wrong optical parameters.
- Optical parameters need to be optimized for analyses of larger particles and finer particles separately, even if the API is same. For example, the same API when analysed as coarse powder and in its micronized form may have different optical parameters even though the entire methods may differ.

III. Data Quality (Background Data)

IV. Precautionary Measures:

- Material should not be stuck to the cell windows
- Sample should not be exposed to atmosphere (especially in the case of hygroscopic material),since that may result in agglomeration.
- In case of hygroscopic material, weighing must be in a dehumidifier room
- There should be no dispersant contamination
- There should be no thermal gradients
- The system should be properly aligned
- The inner detector data should be free from castellation (for dry method)

V. Sample Data

VI. Precautionary Measures :

- There should be reasonable signal-to-noise levels
- There should be no multiple scattering
- There should be no negative data
- There should be no noisy data

VII. Distributions with distinctly different modes of occurrence (disconnected peaks) can indicate issues such as bubble peaks, thermal artifacts, dry dispersion artifacts, opalescent or reflective artifacts, and optical model artifacts. Insufficient degassed dispersant may lead to a bubble peak. Another reason for disconnected peaks is thermally unstable dispersant in circulation unit within the system. Hence sufficient time should be given to the dispersant to circulate into the system to achieve thermal equilibrium.

III. Method Development :

- It is recommended that the nature of sample be studied in detail before starting the actual method development. The following checklist needs to be considered:
  - Does the sample consist mainly of larger particles?
  - Does the sample consist mainly of small particles or of micronized particles?
  - A sample that consists mainly of smaller particles may have an inherent problem of high static charges between particles and, as a result, agglomeration can occur.
  - Shape of the particle may impact the result. Hence, before initiating the method development, the method developer should examine the particles under a microscope (Figures 1 and 2).
  - Dry spread or oil can be used to prepare a few slides. Although there may be challenges due to agglomeration, the shape of the basic particle needs to be understood very clearly.

- Elongated or needle-shaped particles are generally very fragile and will break at the slightest pressure. It should be noted that any type of sample preparation for laser diffraction analysis involves different levels of energy being imparted to the sample to remove agglomeration. These external energies, like sonication in wet method or air jet in dry method, could lead to the breaking down of the primary particle leading to an entirely wrong evaluation.

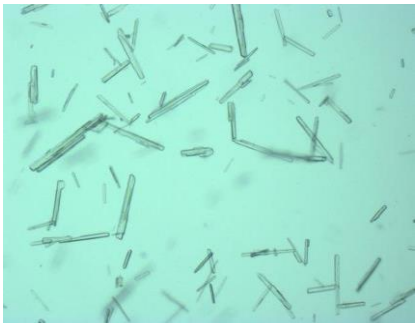


Fig 1: Microscopic image of needle-shaped fragile crystals

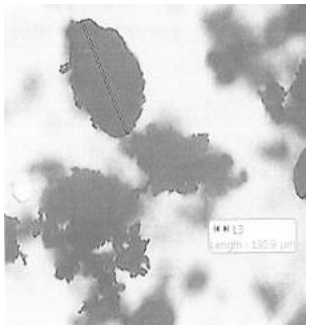


Fig 2: Particles in the form of agglomers

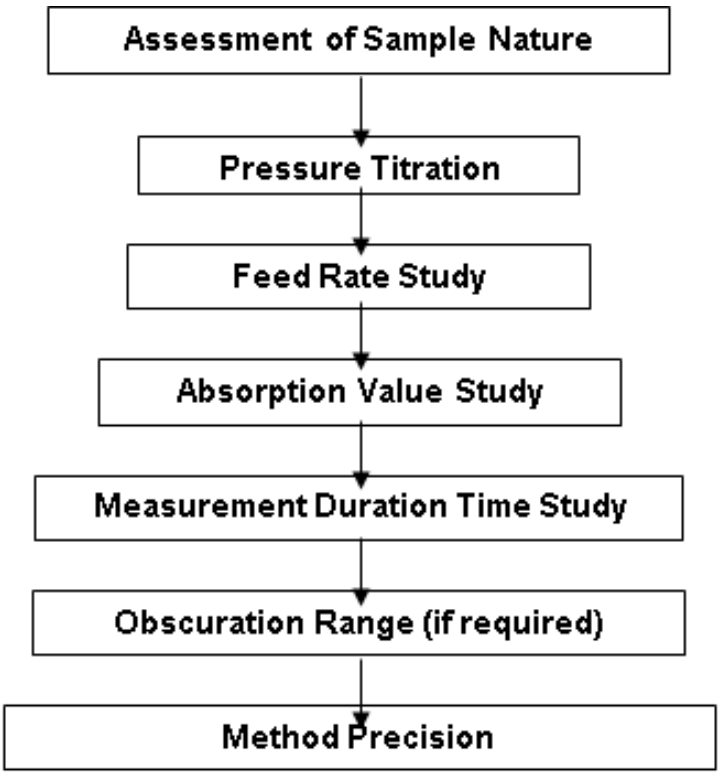
- Crystalline and amorphous particles have different flow properties and mechanical strengths. While choosing the dispersion method, this aspect should be considered.
- Wet or dry dispersion method is an important matter for consideration and decision. A dry dispersion method has the advantage that the sample is analysed “as-is” without addition of any other chemical. Thus there is little chance of any interaction in which the nature of the particle can change.
- Alternative techniques like microscope, dynamic light scattering, specific surface area, etc., should be evaluated in order to get a complete picture of the particle characterization.
- For obtaining samples for most pharmaceutical materials, especially poly-disperse powders, several factors must be considered, e.g., quantity of powder from which sample needs to be obtained, degree or tendency of segregation, etc.
- Statistical sampling is a necessity and as a thumb rule, the larger the particle size and more poly-disperse the sample, the larger should be the sample size withdrawn for analysis in order to compensate for error and variation of result.
- When taking a sample for a measurement it is most important to ensure that the specimen sample is representative of the whole sample
- Mie theory or Fraunhofer theory: Mie theory essentially considers the particles to be transparent or translucent. To account for the transparency of the particles we need to know the optical properties of the material.
    - Real refractive index of the particle
    - Imaginary refractive index of the particle
    - Refractive index of the medium
- Fraunhofer theory considers the particles to be opaque. Under this theory, there is no scope of incorporating these above-mentioned properties.

- An initial review of the particles under a microscope can immediately help in choosing the correct approach. Generally, Mie theory is a better approach for smaller particles (<40 microns), wherein most of the pharmaceutical actives are used.
    - Optical Parameter
    - Refractive Index of Dispersant
    - Refractive index and absorption property of Particle
    - RI required only up to 2 decimal places.
    - Imaginary RI (Absorption) required 0.001, 0.01, 0.1, and 1.0.
    - Coloured particles absorb the laser light hence absorption is 1.0 or above.
    - Blue light: The option of blue light/secondary wavelength is provided to elicit scattering from sub-micron particles (<1 micron). Some equipment has the option of providing blue light analysis/secondary wavelength. It is recommended to keep the blue light "ON" during acquisition of data so that it can be evaluated later.

I. Dry Method Development:

- Some samples can only be measured in dry state as they react with all wet dispersants.
- For example, particles may dissolve or may swell when in contact with liquid. Another consideration is whether the sample in its dry state is free flowing. Highly cohesive sample nature is not suitable for dry dispersion method as the sample may clump together and give biased measurements.

General workflow for method development for Particle Size Determination (Dry Dispersion):



- **Advantages in using dry method instead of wet method:**
  - ❖ There is no chance of interaction with any dispersant as air is used as dispersant.
  - ❖ Quicker analysis time than wet.
  - ❖ Less sample preparation time.
  - ❖ Larger mass is analysed in dry method, which leads to better sampling and reproducible results for a coarse material.

- **Disadvantages in using dry method**
  - ❖ All samples are not suitable for dry method.
  - ❖ Less suitable for use with very fine or cohesive materials, extremely fragile materials like needle-shaped particles, where large quantities of samples are not available or are very expensive.

a. **Air Pressure :**

- ❖ Air pressure is used to reduce the adhesion force between particles. A lower than optimum pressure will lead to high agglomeration while a higher pressure can break the primary particle. In some instruments more than one vent is available, so it is advisable to start with the one with the lowest energy. Pressure titrations should be carried out to find out the optimum air pressure required to disperse the sample.
- ❖ Study Design: The state of dispersion shall be assessed by performing a single measurement at each pressure interval (i.e. 0.5 bar, 1.0 bar, 1.5 bar, 2.0 bar, 2.5 bar, 3.0 bar, 3.5 bar, 4.0 bar). Air pressure for analysis shall be selected from the pressure region over which particle size is nearly constant over a range of pressure intervals.

b. **Feed Rate:**

- ❖ Feed rate controls the rate at which the material is fed through the vent to the measurement cell. The feed rate must be set so that the obscuration is in the appropriate range for most of the measurement duration. In addition to feed rate vibrations, the tray level can also be adjusted using levelling screws to control the flow rate of the sample into the hopper.
- ❖ Study Design: Variation in feed rate should be studied by performing a single determination at each feed rate level (i.e., 40%, 50%, 60%). The feed rate should be selected with the help of visual observation for sample feeding as well as by comparing the data obtained at each level. Factors such as impact of slit width, hopper gap, number of ball bearings present inside the sieve and the type of sieve (fine or coarse) should be assessed by performing a single determination for each parameter.

c. **Absorption Value: (If applicable under Mie Theory):**

- ❖ Absorption value study shall be performed by changing the absorption value so that the weighted residual is at minimum or the peak shape is satisfactory. For this purpose, absorption values generally used are 1, 0.1, 0.01, 0.001, etc.

- Study Design: Initially measurement should be performed with absorption value as 0.1. Then the method should be assessed at each absorption value by performing duplicate measurements. The suitable absorption value should be selected according to sample properties, the nature of the histogram and the weighted residual value.

d. Measurement Duration:

- For larger particles, segregation can occur on the sample feeder tray due to vibrations. Thus, measurement time should allow most, if not all, of the sample kept on the tray to be measured. Larger particles typically would require longer time for a representative measurement. A good starting point is 5 seconds.
- Study Design: Initially, the measurement duration should be selected such that the sample should be present inside the measurement cell for this duration. Duplicate determinations at the selected measurement duration and single determinations at higher and lower levels of measurement durations should be performed. Higher and lower levels can be selected depending on the analysis trend obtained.

e. Obscuration Range:

- Generally, the default obscuration range for dry dispersion using the Malvern Mastersizer is 2-6%. However, depending upon sample flowability, sample size and sample nature, this range should be modified if required.
- Cohesive, fine samples will need to be measured at lower obscuration to maximize the efficiency of the disperser, whereas coarser materials should be measured at higher obscuration. If poor dispersion is observed for fine or cohesive materials, then the upper obscuration limit should be reduced to improve the efficiency of the disperser. In case of static material, maximum air pressure can be applied in between measurements to avoid any carry over from the previous analysis.
- Choice of sample size also plays a crucial role for achieving the suitable obscuration range and in obtaining reproducible results. Different types of sampling trays (macro and micro) should be used according to availability of sample quantity. Recommended obscuration ranges dependent on particle size are:

Particle size	Obscuration range
Fine particles	0.5-3%
Coarse particles	2-6%

Study Design: Since in dry dispersion, obscuration cannot be controlled through addition of sample to the dispersion unit, an obscuration filter should be applied for the selected obscuration range. This parameter shall be evaluated only if any abnormal observation is recorded during measurement with default obscuration range. For this purpose, a single determination shall be performed using the selected obscuration range.

f. Precision:

- The precision of the laser diffraction measurements should be assessed using relative standard deviation. Acceptance criteria for the precision of the method should be as below:

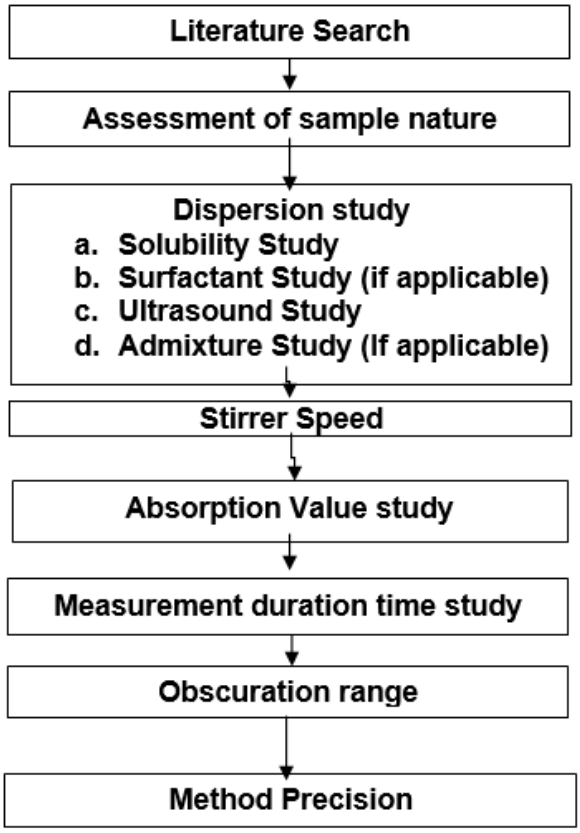
Particle size value	%RSD limit for d (0.1)	%RSD limit for d (0.5)	%RSD limit for d (0.9)
Mean observed particle size value of d (0.5) $\leq$ 10µm	NMT 30%	NMT 20%	NMT 30%
Mean observed particle size value of d(0.5) > 10µm	NMT 15%	NMT 10%	NMT 15%

Study Design: Method precision should be demonstrated by preparing and analyzing six samples as per the selected parameters, representing a single batch. Values of d (0.1), d (0.5) and d (0.9) should be determined and the precision should be evaluated by computing the relative standard deviation for these values of six samples.

II. Wet Method Development:

- The first point to consider when measuring a sample using the wet method is the choice of dispersant (suspension medium).

General workflow for method development for Particle Size Determination (Wet Dispersion):



- **Advantages of using Wet method**
  - Samples with high cohesion are easily dispersed.
  - Typically, smaller sample quantity is required.
  - However, not all samples are possible for use with wet method, e.g., where the sample reacts with the dispersant, like swelling or changes in polymorphic forms in some extreme cases.
  - Large sized polydisperse samples are unsuitable for the wet method

- **Choice of Dispersant**
  - Dispersion should be prepared in media in which the sample is insoluble.
  - Wetting of the material should be appropriate.

- **Examples of dispersant**
  - De-ionized water, organic acids, alcohols (methanol, ethanol, IPA), simple alkanes (hexane/heptane/iso-octane/cyclohexane/isopar-G)
  - Mixture of more than one component (sunflower oil, liquid paraffin). Sunflower oil is a natural product and its quality is not exactly replicable; hence, it is better avoided.

a. Dispersant Choice

- For successful laser diffraction measurement, a dispersant should be chosen such that:
- It is chemically compatible with the materials used in the instrument.
- It is free from bubbles (proper degassing should be done) and other particles.
- It has a refractive index different from that of the particles.
- It has suitable viscosity.
- Mixing sample and dispersant in a beaker and observing the resulting suspension is a good way of checking wettability, a crucial feature. (Please refer to Figure 3). Good wetting produces a uniform suspension and provides easy and stable particle dispersion. Since wetting characteristics depend on the surface tension between the particles and the liquid, this can be improved by using a surfactant. However, too much surfactant can cause foaming and bubbles which may be interpreted as large particles.

Fig 3: Homogenous and heterogeneous suspension (settling of sample)

b. Dispersion Preparation

- It is necessary to break down the inter-particle forces. The particles should be fully wetted.
- No visible change should take place as can be checked by:
    - Dissolution
    - Aggregation
    - Settling
- The dispersion must be stable, i.e., the sample should not dissolve or agglomerate in the chosen dispersion medium.
- Vortexing or external sonication may be used for proper dispersion.
- Appropriate surfactants should be tried out as required.

- Examples of surfactants
    - Igepal CA-360, Tween 20/80, Span 20/80/85, Triton X 100
    - Sodium Lauryl Sulphate, Sodium-bis-2-etheylhexylsulfosiccinate (AOT).
    - Cetyl Triethyl Ammonium Bromide (CTAB)
    - Docusate Sodium (DOSS)
    - Lecithin
- Optical Parameter
- Refractive Index of Dispersant
- Refractive index and absorption property of Particles
- RI required only up to 2 decimal places.
- Imaginary RI (Absorption) required 0.001, 0.01, 0.1, and 1.0.
- Imaginary RI (Absorption) for surface roughness and internal structure
- Milled product – Many edges-lose light-hence absorption is 0.1 or above.
- Coloured particles absorbs the laser light hence absorption is 1.0 or above.

c. Sonication

- Breakage of primary particles due to high stirrer speed or sonication should be studied under microscope. Sonication time should be optimized by conducting a dispersion titration. Readings should also be taken after stopping the ultrasound so as to check whether re-agglomeration is occurring.
- Study Design: Initial solubility study should be performed using various dispersants and the dispersants in which the sample is insoluble should be selected for further study. In the next step, wetting of the sample by the dispersant should be evaluated visually. If needed, addition of surfactant should also be done to get the satisfactory dispersion.
- Application of ultrasound should improve the state of dispersion. If ultrasound is applied for improving dispersion, its effect should be evaluated by performing single determinations at the sonication intervals of 30 seconds, 1 minute, 2 minutes, 3 minutes, and 4 minutes.
- Stirring: A stirring speed should be set appropriate in bringing the entire dispersed sample through the sampler. It should not be too low so that the bigger particles cannot reach the analysis area, nor too high which may generate bubbles. A good starting point is about 1500 RPM. For heavier liquid, the use of higher RPMs (2000 or more) should be evaluated.
- Study Design: Initially, measurements should be performed with stirrer speed value set at 2000rpm, after which the method should be assessed at each stirrer speed value by performing duplicate determinations. The suitable stirrer speed value should be selected according to sample properties and the nature of the histogram.

d. **Absorption Value: (If Applicable under Mie Theory):**

- Refer dry method
- Study Design: Refer dry method

e. **Measurement Time:**

- Typically, one should allow for longer time for samples of larger particle sizes to allow for enough time to measure all the particles in the sample. A good starting point is 10 seconds. It can go up to 20 seconds or more, if required, when more viscous dispersant is being used.
- Study Design: Initially, measurement duration should be selected in such a way that sample should be present inside the measurement cell for this duration. Duplicate determinations at the selected measurement duration and single determinations at higher and lower levels of measurement duration should be performed. Higher and lower levels can be selected depending on the analysis trend obtained by the data.

f. **Obscuration Range:**

- Enough samples should be measured to get a stable scattering signal data and sufficient signal-to-noise ratio. For this purpose, sample weight in the dispersion should be such that obscuration is easily achieved by adding the dispersion to the system. One must avoid the use of dropper for pouring the sample suspension into the dispersant unit, as this may lead to the erratic results (please refer to Figure 4). If the sample is withdrawn from the upper portion of the sample suspension, then lower results may be observed. The suspension should be poured into the dispersant unit and shaken vigorously.

While the addition of enough dispersion (sample) is required to get a sufficient signal-to-noise ratio, too much sample addition may result in multiple scattering; i.e., if the concentration of particles in the cell is too high, there is a higher probability that the laser light has been scattered by more than one particle before hitting the detector. Measurements of fine particles are more likely to be affected by multiple scattering whereas measurements of coarse particles are more likely to be affected by sampling or sample nature.

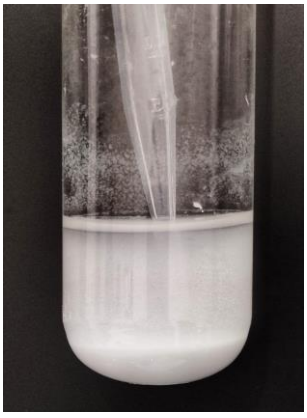


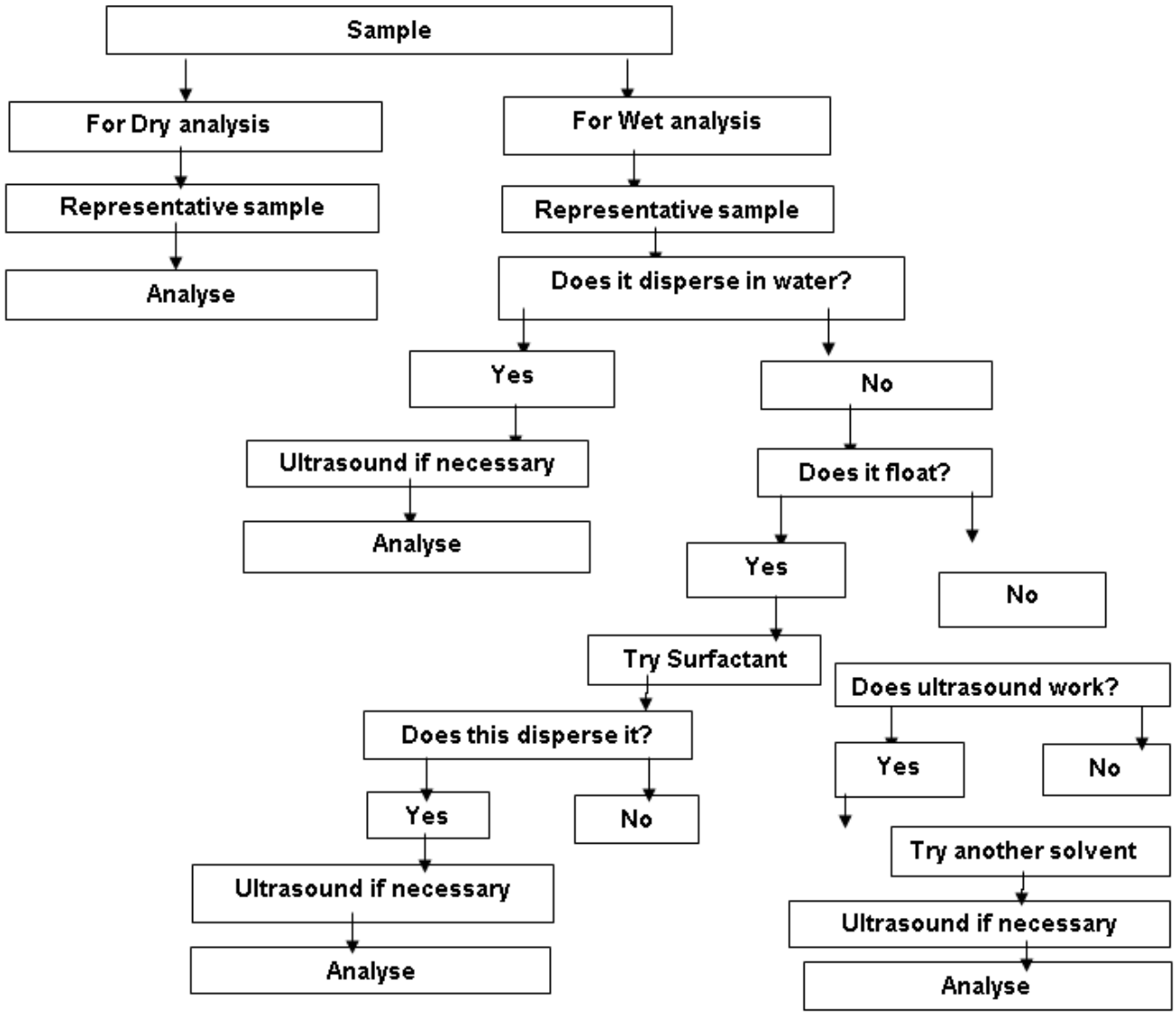
Figure 4: Use of dropper

Recommended obscuration ranges dependent on particle size are:

Particle size	Obscuration range
Fine particles	~5 to 10% (less than 5% may be required for <1µm)
Micronized or milling particle	10-20%
Coarse particles	15 to 20%
Polydisperse samples	20 to 25%

Study Design: Obscuration range should be fixed in such a way that enough sample is present inside the measurement cell and multiple scattering effects are not observed. For this, an obscuration range should be selected according to the nature of the sample, and then a precision study at minimum obscuration and maximum obscuration level should be performed. For example, if the obscuration range is selected as 5 to 12% then precision studies at about 5% obscuration and about 12% obscuration should be performed.

g. Summary of Sample Preparation (Dry and Wet Method)



- Precision: Refer dry method
- Study Design: Refer dry method

h. Stability Of Dispersion:

- Measurements of the prepared dispersion should be taken over different time intervals in order to understand the stability of dispersion prepared. The data should be recorded at different time intervals, i.e. after 1min, 3min, 5min, 7min, and 10min.

IV. Method Validation :

- USP General chapter <1225> is a good starting point for method validation parameters.
- Method accuracy is not normally conducted for PSD. This is because the particle size determined by laser diffraction assumes that the particles are purely spherical, which in reality is incorrect. The accuracy of the instrument is established during a qualification process with respect to traceable standards.

6 Abbreviations

USP	:	United States Pharmacopeia
API	:	Active pharmaceutical ingredient
PSD	:	Particle size distribution
LNB	:	Lab note book
ISO	:	International Organization for Standardization
e.g.	:	As for example

7 References

- ISO 13320, Malvern Manual, USP<429> and EP <2.9.31>

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