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Impurity Profiling And Degradation Study: A Review

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Abstract:

In the pharmaceutical industry, an impurity is considered as any other organic material, besides the drug substance, pharmaceutical ingredients arising during synthesis, or unwanted chemicals that remains with APIs. The impurity may be developed either during formulation or upon aging of both APIs and formulated APIs in medicines. The presence of these unwanted chemicals, even in small amounts, may influence the efficacy and safety of pharmaceutical products. The highly sophisticated instrumentation, such as mass spectrophotometer attached to the gas chromatography or HPLC in various matrices. Impurity profiling includes identification, structure elucidation, and quantitative determination of impurities and degradation products in bulk drug materials and pharmaceutical formulations. The advanced hyphenated technique has revolutionized any impurity profiling, by not only separation but structural identification of impurities as well among all techniques. Impurity profiling study has been in the limelight in the recent pharmaceutical scenario and its importance is increasing day by day. The present article reveals different impurities found in the APIs. The methods for identifying them and the possible measures to deal with the interferences caused by them in pharmaceutical analysis.

Keywords:

Impurity profiling, Degradation products, Analytical identification methods, Structural elucidation, Characterization, ICH guidelines, Hyphenated methods, and Sources of impurities.

Abbreviations:

1) DP – Degradation products
2) ICH – International Conference on harmonization
3) IP – Indian Pharmacopeia
4) API – Active pharmaceutical ingredients
5) ppm – parts per million
6) PDE – Permitted daily dose
7) US-FDA – United states food and drug administration
8) NDA – New drug application
9) ANDA – Abbreviated new drug application
10) TGA – Therapeutic governance authority, Australia
11) NCE – New chemical entity
12) BP – British pharmacopeia
13) LOD – Limit of detection

14) LOQ – Limit of quantitation
15) NMR – Nuclear magnetic resonance
16) FT-ICR – Fourier transform ion cyclotron resonance
17) MS – Mass spectroscopy
18) HSQC – Heteronuclear single quantum coherence
19) HMBC – Heteronuclear multiple bond correlation
20) Q-TOF – Quadrapole time-of-flight

Introduction [3]: -

The regulatory requirements for Impurity profiling and degradation study of drug substances and the drug product have been increased for pharmaceutical drugs and products. The regulatory bodies are becoming more strict in controlling and maintaining the quality and purity of drug substances because of impurities or degraded products beyond the prescribed limit.

Impurity: An impurity of a drug substance is as per ICH guidelines Q3A "*Any component of the drug substance that is not the chemical entity defined as the drug substance*" [1]

As per ICH guideline Q3B, Impurity in a drug product is "*any component of the drug product that is not the chemical entity defined as the drug substance or an excipient in the drug product.*" [2]

Impurity can be of three types: - (1) Impurities closely related to the product and coming from the chemical or from the biosynthetic route itself, (2) Impurities formed due to spontaneous decomposition of the drug during the storage or on exposure to extreme conditions, (3) the precursors that may be present in the final product as impurities. [3]

Impurities present in more than 0.1% should be identified and quantified by selective methods.

Impurity profiling: Impurity profiling may be defined as *"the common name of analytical activities with the aim of detecting, identifying and/ or elucidating the structure and quantitatively determining organic, inorganic impurities and residual solvents in bulk drug and pharmaceutical formulations."* [1-5]

It includes the identity or some qualitative analytical designation, the range of each impurity observed, and the type of each identified impurity. The impurity profile of a substance under investigation gives the maximum possible types of impurities present in it. The Impurity profile is normally dependent upon the process or origin of the API.

Degradation product: The degradation products are nothing but impurity. Degradation products are defined as *"a molecule resulting from a change in the drug substance brought about over time."*

Degradation profile: A description of the degradation product observed in the drug substance or product.

Process contaminants: Process contaminants are identified or unidentified substances, including reagents, catalysts, and other inorganic impurities; and may also include foreign substances.

Specified impurity: An impurity that is individually listed and limited with a specific acceptance criterion in the new drug substance specification. A specified impurity can be either identified or unidentified.

Unidentified Impurities and unidentified Degradation products: Impurities or degradation products for which structural characterization have not been achieved and that is identified solely by qualitative analysis properties.

Unspecified impurities and unspecified Degradation products: Impurities or degradation products that are limited by general acceptance criteria but not individually listed with their specific acceptance criteria in individual monographs.

Foreign substances (Extraneous contaminants): An impurity that arises from any sources extraneous to the manufacturing process and that is introduced by contamination or adulteration.

Related substances: Related substances are structurally related to a drug substance. These substances may be (a) identified or unidentified impurities arising from the synthesis manufacturing process, or (b) identified or unidentified degradation products that result from drug substance or drug product manufacturing process.

Stereometric Impurity: A compound with the same 2-dimensional chemical structure as the drug substance but differs in the 3-D orientation of substituents at chiral centres within that structure.

Toxic Impurities : Toxic impurities have significant undesirable biological activity, even as minor components, and require individual identification and quantification by specific tests.

Concomitant components: The Concomitant components are the characteristics of many drug substances and are not considered to be impurities in the pharmacopeia sense. Any component that can be considered a toxic impurity because of a significant undesirable biological effect is not considered to be a concomitant component.

Classification of Impurities [6-8]: Impurities are classified as per ICH guidelines;

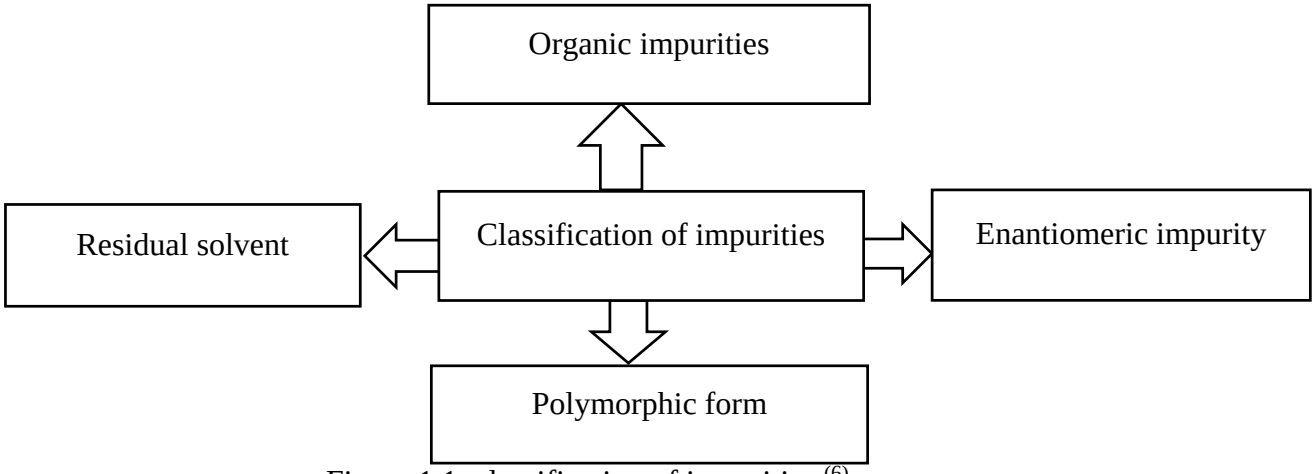


Figure 1.1: classification of impurities [6]

1) **Common names:** [9-12]
   a) By-products
   b) Degradation product
   c) Interaction products
   d) Intermediates
   e) Penultimate intermediates
   f) Related products
   g) Transformation products

a) **By-products:** The compound produced in the reaction other than the required intermediates. They can occur through a variety of side reactions, such as overreaction, incomplete reaction, demonization and rearrangement, and an unwanted reaction between starting materials or intermediates with chemical reagents or catalysts.

b) **Degradation product:** They are formed by the decomposition of active ingredients or other materials of interest by the effect of external factors like heat, light, and moisture.

c) Interaction products: These products are intentionally or unintentionally formed in the interaction between various chemicals involved.

d) Intermediates: The compounds produced during the synthesis of the desired material or as a part of the route of synthesis.

e) Penultimate intermediates: It is the last compound in the synthesis chain before the production of the final desired compound.

f) Related products: These are chemically similar to drug substances and may even possess biological activity.

g) **Transformation products:** They are related to theorized and non-theorized products that can occur in a reaction. They are similar to by-products except that more is known about these reaction products.

2) **United State Pharmacopeia[6,9]:** The United State Pharmacopeia (USP) classified impurity in various sections;

- Impurities in official articles
- Ordinary impurities
- Organic volatile impurities

3) **ICH Terminology[1-5]:** According to ICH guidelines $Q3_A$ and $Q3_B$, impurities in the drug substance can be classified as :

a) **Organic impurities:** These can be formed during the manufacturing process or during the storage of new drug substances. They can be identified or unidentified, volatile or non-volatile. This includes starting materials, intermediates, by-products, degradation products, reagents, ligands, and catalysts.

b) **Inorganic impurities:** These can arise during the manufacturing process; normally they are known and identified. This includes reagents, ligands, catalysts, heavy metals, other residual metals, inorganic salts, and other materials like filter aids, and charcoal.

c) **Residual solvents [13]:** These are generally inorganic/organic liquids that are used during the synthesis of drug substances as a vehicle for the preparation of solution or suspension. Appropriate control of residual solvents is necessary since they have known toxicity. ICH Q3C (5) guideline provides the limits of residual solvent based on existing safety and toxicity data. These were classified into three categories:

1) **Class 1 (the most toxic and/or environmentally hazardous):** these are highly toxic and are limited to 2-8 ppm, for environmentally hazardous chemicals like trichloroethane the limit of 1500 ppm is applied.
2) **Class 2 (considered a lesser risk):** These should be limited in their usage. Two different approaches were described in guidelines for setting limits of class 2 solvents. The first approach is used when the permitted daily dose (PDE) cannot be estimated; concentration limits are calculated based on daily intake of theoretical product mass of 10 gm.
3) **Class 3 (the lowest risk category):** These have low toxic potential and are limited to 5000 ppm (0.5%w/w).

ICH limits for Impurities: According to ICH guidelines on impurities in new drug products; identification of impurities below 0.1% level, is not considered to be necessary unless potential impurities are expected to be unusually potent or toxic [1-5].

Identification and Qualification threshold of impurities and degradation products: ICH guidelines Q3A and Q3B provide the reporting, identification, and qualification threshold of impurities and degradation products. The thresholds are slightly different for both as ICH treats degradation products differently than impurities, although degradation products are impurities only.

Table 1: Thresholds for reporting impurities [3,13]

Maximum daily dose	Reporting threshold	Identification threshold	Qualification threshold
Less or equal to 2gm/day	0.05%	0.1% or 1 mg/day (Whichever is lower)	0.15% or 1 mg/day (Whichever is lower)
>2gm/day	0.03%	0.05%	0.05%

Table 2: Threshold for reporting degradation products [3]

Maximum daily dose	Reporting threshold	Identification threshold	Qualification threshold
≤ 1 mg		1% or 5µg TDI	0.15% or 1 mg/day
1 mg – 10 mg		0.5% or 20µg TDI	0.05%
10 mg-100 mg			0.5% or 200µg TDI
<10 mg			1% or 50µg TDI
>10mg-2 gm		0.2% or 2 mg TDI	
>100 mg-2 gm			0.2% or 3 mg TDI
≤ 1 gm	0.1%		
> 1 gm	0.05%		
>2 gm		0.1%	
>2 gm			0.15%

Impurities commonly found in medicinal preparation: [6]

- Activity depressing impurities
- Due to colouring or flavouring substances
- Humidity
- Decrease shelf life
- Physical and chemical properties
- Impurities due to which substances become incompatible

Regulatory guidelines on impurities in Active Pharmaceutical Ingredients: Ethical, economic, and competitive reasons, as well as those of safety and efficacy, support the need to monitor impurities in drug products. However, monitoring impurities and controlling these impurities means different things to different people or the same people at different times, even those in the pharmaceutical sciences and industry. The United States Food and Drug Administration (US FDA) has endorsed the guidance prepared under the guidance of the International Conference on Harmonization (ICH). The guidelines not only aid the sponsors of New Drug Application (NDA) or Abbreviated New Drug Application (ANDA) with the type of information that should be submitted with their applications but also assist the FDA reviewers and field investigators in their consistent interpretation and implementation of regulations.

The various regulatory guidelines regarding impurities are as follows [1-5,14]

1) ICH guidelines “stability testing of new drug substances and products”- Q1A
2) ICH guidelines “Impurities in new drug substances”- Q3A
3) ICH guidelines “Impurities in new drug products”- Q3B
4) ICH guidelines “Impurities: Guidelines for residual solvents”- Q3C
5) US-FDA guidelines “NDAs – Impurities in new drug substances”
6) US-FDA guidelines “ANDAs – Impurities in new drug substances”
7) Australian regulatory guideline for prescription medicines, Therapeutic Governance Authority (TGA), Australia

Sources of Impurities [3,15-50]:

A) **Crystallization-related impurities:** Based on the realization that the nature of structure adopted by a given compound upon crystallization could exert a profound effect on the solid-state properties of that system, the pharmaceutical industry is required to take a strong interest in polymorphism and solvatomorphism as per the regulations laid down by the regulatory authorities. *If a substance has the same elemental composition but can exist in different crystal packing arrangements, is called polymorphs and the phenomenon is called polymorphism. If the substances exist in different crystal packing arrangements with different elemental compositions, the phenomenon is known as solvatomorphism.*

B) **Stereochemistry-related impurities:** Stereoisomers can be considered an impurity in drug substances, although ICH excludes stereochemical impurities, pharmacopoeias considered them as an ordinary impurity. Chiral molecules are frequently called enantiomers. The single enantiomeric form of the chiral drug is now considered as an improved chemical entity that may offer a better pharmacological profile and an increased therapeutic index with a more favourable adverse reaction profile. However, the pharmacokinetic profile of levofloxacin (S-isomeric form) and ofloxacin (R-isomeric form) are comparable, suggesting the lack of advantages of a single isomer in this regard. The prominent single isomer drugs, which are being marketed, include levofloxacin (S-ofloxacin), levalbuterol (R-albuterol), and esomeprazole (S-omeprazole).

C) Residual solvents: Residual solvents are organic volatile chemicals used during the manufacturing process or generated during production. Some solvents that are known to cause toxicity should be avoided in the production of bulk drugs. Solvents such as benzene (Class I, 2 ppm limit) and carbon tetrachloride (Class I, 4 ppm limit) are to be avoided. On the other hand, the most commonly used solvents such as methylene chloride (600 ppm), methanol (3000 ppm), pyridine (200 ppm), toluene (890 ppm), N, N-dimethylformamide (880 ppm), and acetonitrile (410 ppm), are of class II. Class III solvents (acetic acid, acetone, isopropyl alcohol, butanol, ethanol, and ethyl acetate) have permitted daily exposures of 50 mg or less per day. In this regard, ICH guidelines for limits should be strictly followed. [13, 45-47]

D) Synthetic intermediates and by-products: During the synthetic process, impurities in a new drug substance or new chemical entity (NCE) mainly originate from raw materials, intermediates, by-products, and solvents. The raw materials used in the synthesis are generally manufactured to a much lower purity level than a drug substance. Hence, they can contain a number of components that can affect the purity or may react with other chemicals used in the synthesis of a drug substance. The impurities can also be produced by the reaction of impurities present in the solvent itself that is used in the synthesis and may range from trace levels to significant quantity. Intermediates formed during synthesis are also not generally purified to a higher level as in the case of drug substances, hence can form impurity in the final product.

E) Formulation-related impurities: The excipients used to formulate drug products can originate potential impurities. In addition, during the process of formulation the drug is subjected to a variety of conditions like heat, shear, etc, that can attenuate undesirable reactions and form degradation products. Hydrolysis or solvolysis generally takes place in solutions and suspensions that lead to degradation. These reactions can also occur in the dosage forms that are in a solid state such as capsules and tablets when water/ another solvent has been utilized for granulation. It provides a ripe situation for hydrolysis and metal catalysis besides contributing its own impurities. Oxidation occurs for easily oxidizable materials if no precautions are taken. Similarly, photochemical reactions are feasible for light-sensitive materials. Lyophilization and vortex mixing are sometimes used during the process of formulation that is considered high-risk operations which can cause impurity formation.

F) Impurities arising during storage: Degradation of the drug substance is one of the main sources of impurities caused by chemical instability of the drug substance under the conditions (e.g., heat, moisture, solvent use, pH, light, etc.) of manufacturing, isolation, purification, drying, storage, transportation, and interactions with other chemical entities in the formulation.

G) Method-related impurities: A known impurity, 1-(2,6-dichlorophenyl) indolin-2-one is formed in the production of a parenteral dosage form of diclofenac sodium if it is terminally sterilized by autoclave. It was the condition of the autoclave method (i.e., 123 + 2^{o} C) that enforced the intramolecular cyclic reaction of diclofenac sodium forming the indolinone derivative and sodium hydroxide. The formation of this impurity has been found to depend on the initial pH of the formulation. The concentration of the impurity in the resultant product in the ampoule exceeds the limit of the raw material in the BP.

H) Mutual interaction amongst ingredients: Most vitamins are very labile and on aging, they pose a problem of instability in different dosage forms, especially in liquid dosage forms. Degradation of vitamins such as folic acid, pantothenic acid, cyanocobalamin, and thiamine does not give toxic impurities; however, the potency of active ingredients drops below pharmacopeial specifications. Because of mutual interaction, the presence of nicotinamide in a formulation containing 4 vitamins (nicotinamide, pyridoxine, riboflavin, and thiamine) causes the degradation of thiamine to a sub-standard level within a 1-year shelf-life of vitamin B-complex injections. The marketed samples of vitamins B-complex injections were found to have a pH in the range of 2.8-4.0. The custom-made formulation in a simple distilled-water vehicle and in a typical formulated vehicle that included disodium edetate and benzyl alcohol was also investigated and similar mutual interaction causing degradation was observed.

I) Functional group-related typical degradation: Ester hydrolysis can be explained with a few drugs *viz* aspirin, benzocaine, cefotaxime, ethyl-paraben, cefpodoxime proxetil. Hydrolysis is a common phenomenon for ester types of drugs, especially in liquid dosage forms *viz* benzylpenicillin, oxazepam, and lincomycin. Oxidative degradation of drugs like hydrocortisone, methotrexate, hydroxyl group directly bonded to an aromatic ring, conjugated dienes, heterocyclic aromatic rings, nitroso, and nitrite derivatives, and aldehydes are susceptible to oxidative degradation. Photolytic cleavage includes an example of pharmaceutical products that are exposed to light while being manufactured as solid or solution, packaged, or when being stored in pharmacy shops or hospitals for use by consumers. Ergometrine, nifedipine, nitroprusside, riboflavin, and phenothiazines are very liable to photo-oxidation. Decarboxylation of some dissolved carboxylic acids, such as p-aminosalicylic acid; shows the loss of carbon dioxide from the carboxyl group when heated. An example of decarboxylation is the photoreaction of ciprofloxacin.

J) Genotoxic impurities: Genotoxic compounds can be carcinogenic to humans due to their ability to induce chromosomal rearrangements and/or genetic mutations. The ICH M7 (15) guideline provides the limits for the control of genotoxic impurities in pharmaceuticals to limit the risk of carcinogenicity.

Table 3: Sources of impurities [30]

Impurity type	Examples
Crystallization-related impurities	*Polymorphism, Solvatomorphism*
Stereochemistry-related impurities	Levofloxacin(S-ofloxacin) Levalbuterol(R-albuterol) Esomeprazole(S-omeprazole)
Residual solvents	Benzene, carbon tetrachloride, dichloro-methane, etc.
Synthetic intermediates and by-products	Impurity profiling of ecstasy tablets by GC-MS and MDMA samples, impurities produced in intermediates via reductive amination route
Formulation-related impurities	Microbial contamination may occur during the shelf life and subsequent consumer use of a multiple-dose product.
Impurities arising during storage	A number of impurities can originate during the storage or shipment of drug products.
Method related impurities	1-(2,6-dichlorophenyl) indolinone
Mutual interaction amongst ingredients	Nicotinamide, pyridoxine, riboflavin, thiamine
Functional group-related typical degradation	Aspirin, benzocaine, cefotaxime
Process-related drug substances	- Organic - Starting material - Intermediate - By-product - Impurity in starting material
Degradation drug product	- Organic products - Degradation products
Degradation of drug substance or drug	- Organic - Excipient interaction

Benefits of isolation and characterization of impurities and degradation products: The identification or structural characterization of impurities or degradation products is beneficial in many ways: [18-20]

1) Provides an understanding of the source, origin, and nature of impurities or degradation products, that can be utilized for their control during drug synthesis and/or formulation development. There are many reported impurities or degradation products that originate from totally unexpected sources. There was an example of the drug nelfinavir mesylate formulation from the market due to conversion of mesylate to genotoxic impurity, ethyl methyl sulfonate formed by the interaction of nelfinavir mesylate with residual ethanol.
2) Impurities or degradation products can be synthesized once the structure has been elucidated, which leads to the availability of a pure impurity standard. This can be used for a) establishing quantitative validation parameters, like calibration curves, LOD, LOQ, and response factor b) spiking study c) establishing safety in in-vitro and in-vivo qualification studies.
3) Side or adverse effects of drug substances and products can be explained based on the structure of impurities or degradation products.
4) Based on a review of available literature or via computational toxicology assessment by using commercial software, the genotoxic potential of particular impurities or degradation products can be assessed.
5) Correlating whether specific impurities or degradation products are a significant metabolite.
6) Establishing drug degradation mechanisms and pathways.
7) To list impurities or degradation products in compendial monographs for reference.
8) To create or add to the library of spectral data of chemical compounds.
9) Environmental mapping of drugs by focusing on their major impurities or degradation products.

10) Addition into structure searchable drug degradation database e.g., pharma D3.
11) Sometimes understanding chemical reactions that lead to the formation of impurity or degradation products, discloses some unusual chemistry that may even result in new chemical or drug leads.

Analytical method development [1-2]:

New drug development requires meaningful and reliable analytical data to be produced at various stages of the development: -

a) Sample set selection for analytical method development
b) Screening of Chromatographic conditions and phases, typically using the linear solvent strength model of gradient elution
c) Optimization of the method to fine-tune parameters parameter related to ruggedness and robustness.

USP S/N= 2 x height x scale to UV/ peak to peak noise

Method for determining or establishing exposure limit:

Residual solvent class

Class A.: Solvent to be avoided, known human carcinogen

Class B.: Solvent to be limited, neurotoxin, teratogenicity

Class C.: Low toxic potential

Limit of class [1-2]

Option 1: Calculated assuming 10 gm orally

Concentration (ppm) = [(1000µg /mg) x PDE]/ Dose

Option 2: By adding the amount of residual solvent

According to ICH guidelines on impurities in new dry products, identification of impurities below 0.1% level is not considered to be necessary, unless potential impurities are expected to be unusually potent or toxic. According to ICH, the maximum daily dose qualification threshold is to be considered. <2 gm/day 0.1% or 1 mg per day intake (whichever is lower) >2gm/day 0.05%

Identification and Structure Elucidation of Impurities and degradation products [15-39,48]

1) Conventional approach

The conventional approach of identification and structure elucidation of unknown impurities and degradation products involves separation, impurity, or degradant enrichment and isolation or synthesis which is followed by spectral analysis.

The list of methods that can be used are:

a) **Solid-Phase Extraction method:** Solid-phase extraction (SPE) is an increasingly useful sample preparation technique. With SPE, many of the problems associated with liquid-liquid extraction can be prevented, such as incomplete phase separation, less than quantitative recoveries, use of expensive, breakable specialty glassware, and disposal of large quantities of organic solvents. SPE is more efficient than liquid-liquid extraction, and yields quantitative extractions that are easy to perform, rapid, and can be automated.
b) **Liquid-Liquid Extraction method:** Liquid-liquid extraction, also known as solvent extraction and partitioning, is a method to separate compounds based on their relative solubilities in two different immiscible liquids, usually water and an organic solvent. It is an extraction of a substance from one liquid phase into another liquid phase.
c) Supercritical fluid extraction: Supercritical fluid extraction (SFE) is the process of separating one component from another, using supercritical fluids as the extracting solvent. Extraction is usually from a solid matrix, but can also be from liquids. SFE can be used as a sample preparation step for

analytical purposes, or on a larger scale to either strip unwanted material from a product or collect a desired product.

d) Column Chromatography: Column chromatography in chemistry is a method used to purify individual chemical compounds from mixtures of compounds. It is often used for preparative applications on scales from mg to Kg. The classical preparative chromatography column is a glass tube with a diameter of 50 mm and a height of 50 cm to 1 m with a tap at the bottom. Two methods are generally used to prepare a column; the Dry method and the Wet method. The individual components are retained by the stationary phase differently and separate from each other while they are running at different speeds through the column with the eluent. At the end of the column, they elute one at a time. During the entire chromatography process, the eluent is collected in a series of fractions.

e) **Flash chromatography:** The technique used most commonly in modern organic research is 'flash chromatography'. In Traditional column chromatography, the rate at which the solvent percolates through the column is slow. In flash chromatography, however, air pressure is used to speed up the flow of the solvent, dramatically decreasing the time needed to purify the sample.

f) **Thin layer chromatography:** Thin-layer chromatography (TLC) is a chromatography technique used to separate mixtures. Thin-layer chromatography is performed on a sheet of glass, plastic, or aluminium foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminium oxide, or cellulose. The different analytes ascend the TLC plates at different rates, separation is achieved. TLC finds many applications to determine the components that are contained in plants. It is also used for monitoring organic reactions and analysing ceramides and fatty acids; for the detection of pesticides or insecticides in food and water; for analysing the dye composition of fibers in forensics and identifying compounds present in a given substance, and for assaying the radiochemical purity of radiopharmaceuticals. To automate the different steps, increase the resolution achieved with TLC, and allow more accurate quantization. This method is referred to as HPTLC (High-performance TLC).

g) **Gas chromatography:** Gas chromatography or gas-liquid chromatography is a common type of chromatography used in analytical chemistry for separating and analysing compounds that can be vaporized without decomposition. Typical uses of GC include testing the purity of a particular substance or separating the different components of a mixture. In some situations, GC may help in identifying a compound. In preparative chromatography, GC can be used to prepare pure compounds from a mixture.

h) **High-performance liquid chromatography:** High-performance liquid chromatography (or high-pressure liquid chromatography, HPLC) is a form of column chromatography used frequently in biochemistry and analytical chemistry, to separate, identify, and quantify compounds, based on their idiosyncratic polarities and interactions with the column's stationary phase. HPLC utilizes different types of stationary phases (typical, hydrophobic, saturated carbon chains), a pump that moves the mobile phase and analyte through the column, and a detector that provides a characteristics retention time from the analyte.

i) **Supercritical fluid chromatography:** Supercritical fluid chromatography (SFC) Is a form of normal phase chromatography that is used for the analysis and purification of low-to-moderate molecular weight, thermally labile molecules. It can also be used for the separation of chiral compounds. Its principles are similar to those of HPLC, however, SFC typically utilizes carbon dioxide as the mobile phase; therefore, the entire chromatography flow path must be pressurized.

j) **Accelerated solvent extraction method:** Accelerated solvent extraction (ASE) is a better technique for the extraction of solid and semisolid sample matrices, using common solvent, at elevated temperatures and pressure. ASE systems are available in the entry-level ASE 150 systems and the fully automated ASE 350. Extraction that normally takes hours can be done in minutes using ASE with pH hardened pathways, using Dionium^{TM} compounds. Compared to techniques such as Soxhlet and sonication, ASE generates results in a fraction of the time. ASE offers a lower cost per sample than other techniques, reducing solvent consumption by up to 90%.

k) **Capillary electrophoresis:** Capillary electrophoresis (CE), also known as capillary zone electrophoresis (CZE), can be used to separate ionic species by their charge and frictional forces, and mass. In traditional electrophoresis, electrical charge analytes move in a conductive liquid medium under the influence of an electric field.

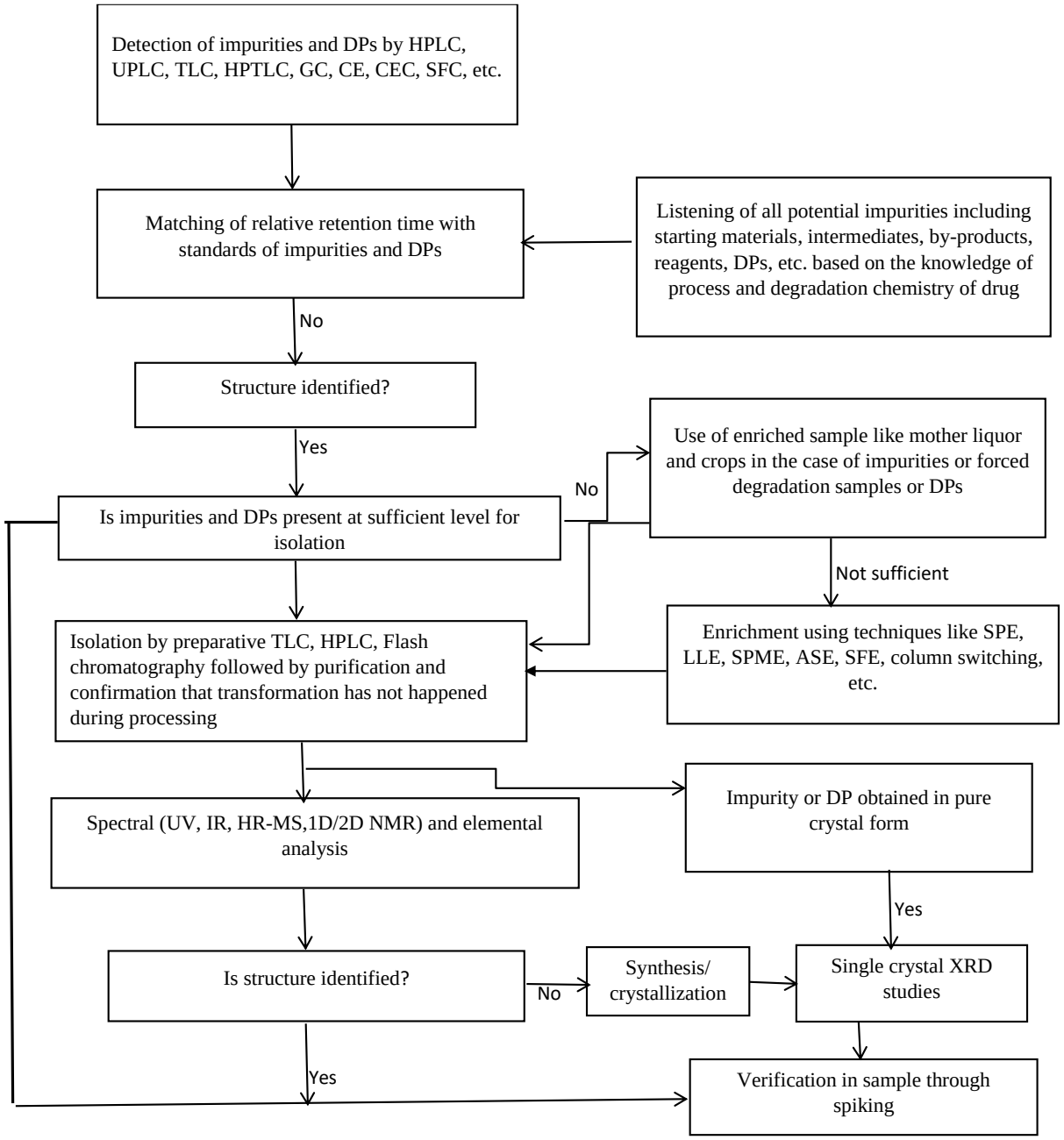
l) **Electron paramagnetic resonance:** Electron paramagnetic resonance (EPR) or electron spin resonance (ESR) spectroscopy is a technique for studying the chemical species that have one or more unpaired electrons, such as organic and inorganic free radicals or inorganic complexes possessing a transition metal ion. The basic physical concept of EPR are analogues to those of NMR, but it is electron spins that are excited here instead of spins of the atomic nuclei. As the most stable molecules have all their electrons paired, the EPR technique is less widely used than NMR.

m) **Gravimetric analysis:** Gravimetric analysis describes a set of methods in analytical chemistry for the quantitative determination of an analyte based on the mass of the solid. A simple example is the measurement of the solids suspended in the water sample. In most cases, the analyte must first be converted to a solid by precipitation, with an appropriate reagent. The precipitate can then be collected by filtration, washed, dried to remove traces of mixture from the solution, and weighed. The amount of analyte in the original sample can then be calculated from the mass of the precipitate and its chemical composition.

n) **UV spectroscopy:** UV spectroscopy is a physical technique of optical spectroscopy that uses light in the visible, ultraviolet, and infrared ranges. The beer-lambert's law states that the absorbance of a solution is directly proportional to the concentration of the absorbing species in the solution and the path length. Thus, for a fixed path length, UV/VIS spectroscopy can be used to determine the concentration of the absorber in a solution. It is necessary to know how rapidly the absorbance changes with concentration.

o) **Infrared spectroscopy:** IR spectroscopy is the subset of the spectroscopy that deals with the infrared region of the electromagnetic spectrum. The infrared portion of the electromagnetic spectrum is usually divided into three regions; the near, mid, and far-infrared, named according to their relation to the visible spectrum. The far-infrared, approximately 400-10 cm^{-1} (1000-30μm), lying adjacent to the microwave region, has low energy and may be used for rotational spectroscopy. The mid-infrared, approximately 4000-400 cm^{-1} (30-2.5μm), may be used to study the fundamental vibrational and associated rotational-vibrational structure.

Figure 1.2: Conventional approach for the characterization of impurities and degradation products (DPs) [18-33]



p) **Fluorescence spectroscopy:** Fluorescence spectroscopy is called fluorometry or Spectro-fluorometry. It is a type of electromagnetic spectroscopy, which analyses the fluorescence from a sample. It involves using a beam of light, usually, ultraviolet light, which excites the electron on the molecules of certain compounds and causes them to emit light of lower energy, typically, but not necessarily, visible light. A complementary technique is absorption spectroscopy.

q) **Mass spectrometry:** Mass spectrometry are used in the industry and academic for both routine and research purpose. Applications of mass spectrometry are

- Used for the analysis of proteins, peptides, and oligonucleotides
- It deals with drug discovery, combinatorial chemistry, pharmacokinetics, and drug metabolism.
- Used in neonatal screening, haemoglobin analysis, and drug testing.
- It also deals with polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), water quality, and food contamination.
- Also used in the oil composition

The instrument of mass spectrometry includes (1) ionization sources, for example, electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI); (2) analyser mass to charge (m/z), for example, quadruple and magnet; (3) FT-ICR detector, for example, the photomultiplier micro-channel plate electron multiplier.

r) **NMR spectroscopy:** NMR spectroscopy is a powerful and theoretically complex analytical tool. In NMR, the chemical environment of the specific nuclei is deduced from the information obtained about the nuclei. Nuclear magnetic resonance (NMR) is a property that magnetic nuclei have in a magnetic field and the applied electromagnetic (EM) pulse or pulses, which cause the nuclei to absorb energy from the EM pulse and radiate this energy back out. The energy radiated back out is at a specific resonance frequency, which depends on the strength of the magnetic field and other factors. This allows for the observation of specific quantum mechanical magnetic properties of an atomic nucleus.

Many scientific techniques exploit NMR phenomena, to study molecular physics, crystals, and non-crystalline materials, through NMR spectroscopy. NMR is also routinely used in advanced medical imaging techniques, such as magnetic resonance imaging (MRI). All stable nuclides that contain an odd number of protons and/or neutrons (see Isotope) have an intrinsic magnetic moment and angular momentum, in other words, a spin > 0, while all nuclides with even numbers of both have spin 0. The most commonly studied nuclei are 1 H (the most NMR-sensitive isotope after the radioactive 3 H) and 13C, although nuclei from isotopes of many other elements are studied by high field NMR spectroscopy as well. A key feature of NMR is that the resonance frequency of a particular substance is directly proportional to the strength of the applied magnetic field. The effectiveness of NMR can also be improved using hyperpolarization, and/or two-dimensional, three-dimensional, and higher dimension multi-frequency techniques.

s) **Raman spectroscopy:** Raman spectroscopy is a spectroscopic technique used to study vibrational, rotational, and other low-frequency modes in a system. It relies on the inelastic scattering or the Raman scattering of the monochromatic light, usually from a laser, in the visible, near-infrared, or near-ultraviolet range. The laser light interacts with the photons or other excitations in the system, resulting in the energy of the laser photons being shifted up or down. The shift in energy gives information about the phonon modes in the system. A sample is illuminated with a laser beam. Light from the illuminated spot is collected with a lens and sent through a monochromator. Wavelengths close to the laser line, due to elastic Rayleigh scattering, are filtered out, while the rest of the collected light is dispersed onto a detector.

There are a number of advanced types of Raman spectroscopy, including surface-enhanced Raman, tip-enhanced Raman, polarized Raman, stimulated Raman (analogous to stimulated emission), transmission Raman, spatially-offset Raman, and hyper Raman. The formation of an impurity band is accompanied by a Fano-type interference for the one-photon scattering. Bands at 1200 and 500 cm^{-1} are observed in Raman spectroscopy for concentrations above 1020 cm^{-3}. They are related to the maxima in the photon density of states and are ascribed to disordered regions or crystalline regions of very small size.

2) Hyphenated methods [6,13]

These are a few limitations of the conventional approach, these are:

a) The process is time-consuming and sometimes becomes complicated if several impurities and degradation products have to be characterized in a single sample.
b) If the impurity or Degradation products formed are present in trace amounts and cannot be enriched, the process becomes more tedious.
c) If an unstable impurity or degradation product is formed, or if there is the possibility of secondary reaction during processing, isolation becomes difficult.

Due to these reasons, hyphenated techniques are the preferred choice for the identification and characterization of impurity or degradation products formed at a trace level. Mostly the available hyphenated instruments have LC, GC, or CE on the front end connected to MS, NMR, or IR on the detection side.

A) MS-based hyphenated techniques [15-33]

- **GC-MS:** It was the first hyphenated technique introduced for the determination of organic volatile impurities, and residual solvents in a sample. However, the volatility and thermal stability of analytes are essential for GC-MS in the characterization of impurities and Degradation products
- **LC-MS:** The most popular hyphenated method for characterization of impurities and degradation products is LC-MS, as it has the potential to give nearly clear structural information about the unknown analyte. This instrument has several advancements and ranges, these are: LC-MS (single quad), LC-MS-MS (triple quad), LC-TOF, LC-MS-TOF (Q-TOF, triple TOFTM), LC-MS-3DTRAP, LC-MS-2DTRAP, LC-Hybrid Trap TOF systems, LC-Orbitrap TM, LC-FTICR (Fourier transform ion cyclotron resonance).
- **CE-MS:** CE (Capillary electrophoresis) and CEC (capillary electro-chromatography) are important techniques for the separation and identification of impurities and degradation products. CEC is a hybrid technique that involves both high efficiency of CE and stationary and mobile phase selectivity of LC. CE/ CEC have been hyphenated with MS for the characterization of impurities and degradation products. This technique is usually restricted to the separation of analytes.
- **SFC-MS:** The technique has the advantage of saving LC solvents but its bench-top instrument was not available commercially for analysis.

B) NMR based hyphenated techniques:

- **LC-NMR:** In 1978 for the first time, the coupling of LC effluent to NMR was reported. To improve the instrument sensitivity and resolution, modern LC-NMR instruments are accompanied by multiple technological advancements, like microprobes, strong field magnets (above 500 MHz), and cryoprobe technology. SPE (Solid-phase extraction) units are embedded in between LC and NMR to overcome the requirement of high volumes of expensive deuterated solvents in the mobile phase. The LC effluent contains low sample concentrations, due to which ^{13}C detection is usually not possible. Also, an insufficient quantity of analyte did not allow the acquisition of heteronuclear HSQC and HMBC spectra. Specific NMR pulse sequences are used to obtain clean spectra free from corresponding residual non-deuterated solvents. Isomers, that generate the same mass and fragmentation pattern, for such compounds LC-NMR data is very useful to confirm their identity.
- **CE-NMR:** If analytes are present in relatively small amounts, hyphenated CE-NMR provides similar advantages as LC-NMR with respect to separation, chemical identification, and structural information. Both continuous and stopped-flow modes, similar to LC-NMR are used in CE-NMR. The typical problem associated with CE-NMR is the shorter residence time of the sample in NMR due to the small sample volume output from CE that affects the detection sensitivity. Although, intensive innovative efforts have been made to improve this.

- **LC-FTIR Systems:** Conventional FT-IR system requires 1–5 mg of sample hence recording becomes difficult when analytes are present or generated in trace quantities or cannot be isolated. Some limitations exist while recording the IR spectrum of impurities or DPs at levels of 0.1% in LC-IR, these include:

- On-line enrichment of analyte is essential.
- Interference of mobile phase components.
- It is difficult to apply chemometrics, especially in the case of gradient elution since the background absorption is strongly influenced by the slight variation in mobile phase composition.
- Complete removal/ elimination of the solvents is difficult.
- Analytes should have low volatility than the mobile phase.
- Differential nature i.e., amorphous or crystalline; of analyte post-deposition and also post solvent elimination.

Interface constitutes the most critical component in LC-IR due to the above-cited reasons. It is available in two types-

(i) Flow cell (on-line)

(ii) Solvent elimination (semi on-line).

On-line LC-IR has limited use and is restricted to major constituents only due to its poor detection limits, while semi on-line has comparatively better sensitivity and gives improved spectral data.

Validation of analytical methods[3,40-41] The validation process involves confirmation or establishing a developed method through laboratory studies, procedures, and systems, which can give accurate and reproducible results for an intended analytical application in a proven and established range. The performance characteristics of the method should meet the requirements of the intended analytical applications and the process can provide documented evidence that the system or procedure does what it is intended for in a systematic, precise, and reliable manner. According to ICH, typical analytical performance characteristics that should be considered in the validation of all the types of methods are:

- **Limit of Detection (LOD):** LOD of an individual analytical method is the lowest concentration/amount of analyte in a sample that the method can detect but not necessarily quantify under the stated experimental conditions. The LOD will not only depend on the procedure of analysis, but also on the type of instrument.
- **Limit of Quantification (LOQ):** LOQ of an individual analytical method is the lowest concentration/amount of an analyte in a sample, which can be quantitively determined with suitable precision and accuracy under stated experimental conditions. The quantification limit is used particularly for the determination of impurities and degradation products.
- **Linearity:** The linearity of an analytical method is its ability to obtain test results, which are directly proportional to the concentration of analyte in the sample.
- **Range:** The range of an analytical method is the between the upper and lower concentration of analyte the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy, and linearity.
- **Robustness:** The robustness is the measure of the analytical method to remain unaffected by small, but deliberate variations in method parameters. It provides an indication of its reliability during normal usage.
- **Ruggedness:** The ruggedness is the degree of responsibility of the results obtained by analysing the same sample under a variety of normal test conditions such as different analysts, instruments, days, reagents, and, columns. The comparison of reproducibility of test results to the precision of the assay is the direct measure of the ruggedness of the method.

Table 4: Goals of impurity investigation [9]

Process-related impurities	Degradation-related impurities
Identify significant impurities	Identify potential degradation products through stress testing and actual degradation products through stability studies
Determine the origin of impurities and the method for elimination or reduction.	Understand degradation pathways and methods to minimize degradation.
Establish a control system for impurities involving: 1) Processing/manufacturing conditions 2) Suitable analytical methods/ specifications	Establish a control system for impurities involving: 1) Processing/manufacturing conditions 2) Suitable analytical methods/ specifications 3) Long term storage conditions including packaging 4) Formulation

Application: Necessary to ensure the safety and efficacy of pharmaceutical products profiling of impurities in drugs is a regulatory expectation. Numerous applications have been sought in the areas of drug designing and monitoring. Quality, stability, and safety of pharmaceutical compounds, whether produced synthetically, extracted from natural products, or produced by recombinant methods.

The applications include alkaloids, amines, amino acids, analgesics, antibacterial, anticonvulsants, antidepressants, tranquilizers, antineoplastic agents, local anaesthetics, macromolecules, steroids, etc.

Table 5: Various Impurities reported in APIs [11-42]

Sr.no.	Drugs	Impurities	Method
1	Amphotericin B	Tetraenes	UV spectroscopy
2	Atropine sulphate	Apo atropine	UV spectroscopy
3	Cloxacillin	N, N-dimethyl	Gas chromatography
4	Dextrose	5-hydroxy methyl furfural	UV chromatography
5	Doxorubicin hydrochloride	Acetone and ethanol	Gas chromatography
6	Ethambutol hydrochloride	2- amino butanol	TLC
7	Fluorescence sodium	Dimethylformamide	Gas chromatography
8	Framicetine sulphate	Ne-amine	TLC
9	Morphine	6-mono acetyl morphine	HPLC
10	10-hydroxy morphine	10-oxomorphine	HPLC
11	Mercaptopurine	Hypoxanthine, 2,5-bis [(N' cyano-N"-methyl) guinidinoethylthiomethyl]-4-methylimidazole	UV spectroscopy
12	Norgestrel	3,17a-diethyl-13-ethyl-3,5-gonadiene-17-ol	TLC, HPLC, and UV spectroscopy
13	Cimetidine	1,8-bis [(N' cyano-N"-methyl) guinidino]-3,6-dithiaoctane	HPLC
14	Celecoxib	[5-(4-methylphenyl)-3-trifluromethyl-1H-pyrazole],4-[5-(2'-methylphenyl)-3-(trifluromethyl-1H-pyrazole-1-yl]-benzene sulphonamide	HPLC, LC, LC-MS-MS
15	Ethynodiol diacetate	17a-ethinyl-ester-4-ene-3a, 17-diol-3-acetate-17-(3'-acetoxy-2'-butenoate), 17a-	HPLC

		ethinylester-4-ene-3a,17-diol-3-acetate-17-(3-oxo-butanoate	
16	Methamphetamine	1,2-dimethyl-3-phenylaziride, ephedrine, methyl-ephedrine, N-formyl-methamphetamine, N-acetyl methamphetamine, N-formyl ephedrine, N-acetyl ephedrine, N, O acetyl ephedrine, methamphetamine dimmer	HPLC
17	Repaglinide	4-carboxymethyl-2-ethoxybenzoic acid,4-cyclohexylaminocarbamoyl methyl-2-ethoxy-benzoic acid,1-cyclohexyl-3-[3-methyl-1,2-(piperidine-1-ylphenyl)-butyl]-urea, 1,3-dicyclohexyl urea	GC
18	Morphine sulphate	5-(hydroxymethyl)-2-furfural	HPLC
19	Budesonide	Impurities or degradation products	HPLC
20	Cefdinir	Related substances	HPLC
21	Donepezil	Process related impurities	HPLC
22	Linezolid	Process related impurities	HPLC
23	Loratidine	Process related impurities	HPLC
24	Rofecoxib	Process related impurities	HPLC
25	Zaleplon	Process related impurities	HPLC

Conclusion:

A pharmaceutical ingredient should pass not only the test such as cGMP, QC, QA tests, water activity but also should qualify for the specified threshold of a new impurity. The impurity profile of pharmaceuticals is receiving increasing importance and drug safety receives more and more attention from the public and from the media. Impurity profiling of a substance under investigation gives the maximum possible account of impurities present in it. Isolation and characterization of impurities are required for acquiring and evaluating data that establishes biological safety which reveals the need and scope of impurity profiling of drugs in pharmaceutical research. Various regulatory authorities like ICH, USFDA, Canadian Drug, and Health Agency are emphasizing the purity requirements and the identification of impurities in active pharmaceutical ingredients (APIs). The key aspect is that the impurity profile of a new chemical entity must be shown to be qualified. With a qualification threshold is 0.1%, or lower for high-dose compounds, the pharmaceutical analyst must give careful thought to their analytical technology.

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