पर्यावरण नमूनों में कीटनाशकों और अन्य कार्बनिक रसायनो का विश्लेषण

Training Course on

Analysis of Pesticides & Other Organic Chemicals in Environmental Samples

05-06 October, 2021

सीएसआईआर-भारतीय विषविज्ञान अनुसंधान संस्थान

CSIR - INDIAN INSTITUTE OF TOXICOLOGY RESEARCH

Central Pollution Control Board (CPCB)
Training Programme on
ANALYSIS OF PESTICIDES AND OTHER ORGANIC CHEMICALS IN ENVIRONMENTAL SAMPLES

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Overview & Environmental sampling techniques

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Since the liberalization in 1992, India has set in motion the irreversible process of opening Indian economy. With changing scenario country is facing multidimensional problems related to environment due to unplanned industrial and urban growth and lack of pollution control measures to mitigate air, water and soil pollution. The increased activities in future need more resources; result in generation of different types of waste materials and will further deteriorate the environment if proper assessment and monitoring through sampling and analysis is not ensured. CPCB and State Pollution Control Boards are making all possible efforts in this regard.

The demand for testing of environmental parameters has grown enormously. As a result, a number of government laboratories and a huge number of private laboratories have entered into the testing business. These laboratories may be recognised by CPCB or state boards or accredited by National Board for Accreditation of Testing and Calibration Laboratories (NABL). The major requirements of the laboratory include infrastructure, instrumentation, facilities and trained manpower. The quality of testing results depends on the proper management and quality check and quality assurance programmes. However the trained manpower is the most important for quality assurance, who is involved in the activities right from sampling to analyses and finalization of testing results. It becomes furthermore important particularly when advance analytical instrumentation techniques are used for the testing of environmental samples for trace analytes including pesticide residues.

Environmental Protection Act 1986 has necessary provisions to protect the environmental quality of the country. Accordingly there are provisions and requirements for sampling and analysis from different environmental components. Therefore it is necessary to collect representative samples from environmental components (water/soil/air/agricultural products etc.) and waste/stack emissions from which they are taken. The sampling team must take due care to avoid contamination of samples at the time of collection, storage and transport so that its concentration remains unchanged till analysis.

There are number of steps in between sample collection to the final results, each of them are equally important, therefore a suitable plan for environmental monitoring should be prepared.

Common steps of the sampling plan and analysis:

- Define objectives of sampling
- Select the parameters for testing
- Mark the sampling location on the site map
- Decide the number of samples to be collected
- Arrange the sampling equipments/ containers/ tools and tackles
- Arrange the safety/ protection equipments for sampling team
- Arrange the transport of team to the site with all necessary items
- Record the site details, date, time and other relevant information
- Collect/ obtain the representative samples
- Appropriate labelling, preserving, storing and transporting of samples
- Deposit and record the samples in sample handing unit
- Processing of samples
- Analysis of samples
- Calculations and data interpretation if needed
- Reporting

Most of the laboratories in our country possess state of the art, latest instruments for sample analysis; however it does not ensure the quality analysis. It has been observed over the time by most of the laboratories and scientists that the weakness of the system for sampling and sample preparation are often responsible for the poor quality of results. Quality results can only be ensured when sample is representative and intact. The outcomes of the analysis are used to take important decisions about the state of environment, industrial and sustainable development and human health. If the results are not satisfactory it is required to repeat the whole procedure including sample collection, storage, transport, sample preparation and instrumental analysis (1).

The hands-on training of sampling team is essential with respect to practical knowledge about safe and uncontaminated collection, preservation, storage and transportation of samples as required for the type of samples and the target analytes. Regular refresher training for newer analytes is also required. Sampling team should have appropriate sample containers, preservatives for different analytes, format to records sampling details (date, time, location etc.) before moving to the sampling site. Team should be properly informed about the site with respect to occupational health and safety protocols while handling waste during sampling and transport. Personal protective equipments should be provided to the sampling team and they should be trained to use the same. The gloves and clothing should be cleaned properly/ replaced before sampling at different places or for different pesticides to avoid contamination (2).

It is necessary to understand the distribution of pollutants with respect to space and time in the environmental component under investigation during the preparation of sampling plan. The spot spills can affect the spatial distribution of contaminants in soils. Vertical stratification in water bodies and mixing of flowing stream can affect their characteristics. Therefore time of sampling becomes important while going for environmental sampling. For example where the effluent quality changes with the cycles of industrial processes over a period of time it should be incorporated in the sampling plan. Similarly when effluent is mixed with the storm water and natural waterways it changes its quality temporarily (1).

Sampling team uses sampling devices and storage containers for sampling. These items should have minimum interaction with the sample. Their regular cleaning should also be ensured for uncontaminated sampling. The containers of glass, polyethylene or fluoropolymer are generally used for sampling as they exhibit minimum interaction with analytical parameters. For example glass containers have been found suitable for samples with trace organics but unsuitable for samples with trace inorganic (2).

The pesticide enters in to the environment when it is used in agriculture (as pesticide and herbicide), for general pest control to protect public health in households and for longer storage of food items and clothes etc. With time the degradation and/or metabolism of pesticide occur. The rates of degradation and dissipation vary considerably according to the type of pesticide and the conditions. Thus pesticide active ingredients, metabolites or breakdown products are present in some component in the environment after its application, spillage or dumping as pesticide residues. Pesticide residue analysis provides a measure of the nature and level of any pesticide within the environment and of its persistence.

Pesticide Sampling

The objectives of the sampling programs and analysis are:

- to investigate residual levels of pesticide in the environment, their movement and their residual rates of degradation
- to identify contaminated areas and/or sources of contamination
- to examine the uptake of pesticides by agricultural components (food, vegetables, flowers, seeds etc.).

Collection of pesticide from Air Environment

Air will be sampled at the rate of 6 to 10 LPM using sorbent tubes with a pre filter (quartz) to collect vapour form of pesticide. Therefore, the collected sorbent tubes after sealing are to be packed in sealed polythene bags and stored properly in ice box filled with ice to avoid any loss of sample due to physical damage or exposure to heat or sunlight to avoid evaporation losses. The samples should be handed over to the lab to be stored properly (at -10^0C) until its processing (3). The samples are then subjected to extraction processes for pesticide residues. The processed samples are analyzed through advance analytical instrumentation techniques.

Water Sampling

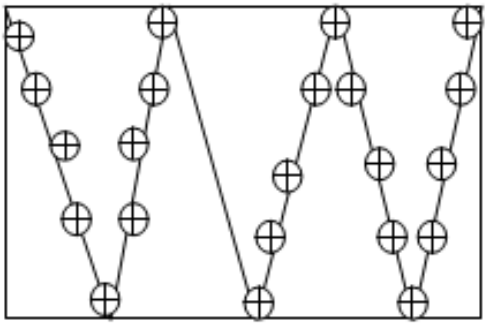
Sampling of water samples from surface water, ground water, effluents etc. need to be dealt carefully depending on the type of sample and the testing parameters. For example when very low concentrations are expected extreme care should be taken to avoid any contamination from containers, lids and collective vessels. The sampling of water samples to analyze pesticide requires extreme care to mitigate contamination and should be followed by its cooling in icebox immediately after sampling for making the concentration intact.

Pesticides are detectable in water for only a short time as these are usually absorbed onto sediments or other organic matter and removed from aqueous solution. Some pesticide residues form a surface film, rather than being dispersed, in this case it is needed to take a surface water sample. Suspended matter in water contains significantly higher pesticide residue than the water itself. Therefore water with suspended matter is collected as water sample. If it is required water can be filtered and analyses of water and suspended matter can be done separately. Handpump/ borewell should be operated for a minimum period of 2-3 min before collecting the ground water sample. As for as possible, properly packed, labelled, intact and uncontaminated samples should reach the laboratory and immediately handed over to the laboratory personal for proper storage and further analysis (1) (2).

Soil Sampling

Sampling of soil, sludge, sediments and any other solids should be carried out as per the specific requirements for particular matrix. For example prior to soil sampling, vegetation and other unwanted matters should be removed from top surface. In case of testing of pesticide in soil samples measures should be taken to eliminate the evaporative losses (1) (2). Pesticide is likely to be confined to top soil in agriculture fields therefore soil samples are collected using a soil auger up to a depth of 15 cm in a W pattern from the field. The collected samples are mixed to form a composite

sample and 500 g of this sub-sample is filled in the sample bag, stored in a clean cool dark place to transport it to the laboratory. The sampling details are recorded in the prescribed format. In case of sediments, the best location for sampling is with little or no flow where fine materials accumulate. When there is a possibility of change in concentration of some contaminants present in the soil samples due to accelerated microbial action in the presence of moisture, the soil sample should be refrigerated ($<6^0$C) while transported to the lab.



Soil Sampling Pattern (W-Shaped)

Soil Auger

Vegetative Tissue Sampling

It is important to collect a representative sample of the area/ product for pesticide analysis. In case of vegetables such as beans, brinjal or any other vegetable crops it is required to take the samples from the four corners of the field and in the centre. Then mix the sample and take a sub-sample out of this sample. Place the representative sub-sample in a clean Crop Nutrition sample bag, label details and store in icebox with ice in it and send to the laboratory to avoid microbial degradation of pesticide residues in the presence moisture and microbes. Approximately 1 kg of tissue is required for pesticide residue analysis.

Sample Processing

Sometimes it is not possible to detect the analyte when it is in trace levels in the original matrix. Therefore in such cases sample preparation through analyte isolation and/ or pre-concentration is required to make it possible to increase analyte concentration to such a high level in the final sample that can be detected by the instrument used for the analysis. Sometimes it is required to simplify the matrix to remove interference by replacing the original matrix with a recipient's matrix, a suitable solvent or gas. It also helps in averaging the sample composition and the prepared concentrated samples can be stored for prolonged period of time than the original sample (1,2).

The variations in the environmental samples may be due to their sampling location, matrix composition and their different states of aggregation. The analyte type in question and their concentration level is also to be considered (1,2). Accordingly the sample preparation method has to be decided. A number of steps are involved in the sample preparation. These steps can be performed at the sampling site during sampling, or on its receipt in the laboratory. However, the processing method is depending on the final determination method. There is a possibility of loss of sample or contamination on different stages of sample preparation. Therefore necessary precautions and measures should be taken to prevent such losses. There is a possibility of random as well as systematic errors in trace analysis. Some of common sources of systematic errors are summarised below (4):

- Differences in volatilities of sample components,
- Adsorption and desorption of chemicals from containers and instruments (memory effects),
- Contamination due to laboratory air and other unknown sources,
- Composition changes caused by addition of chemical reagents,
- Training and expertise of the analyst.

The common steps for preparation of environmental samples to carryout trace level analysis include drying, grinding, homogenization/ mixing, preservation, sieving, purification and removal of interferences, sample fractionation and partitioning. The calibration and verification of the instruments and methods is essential before putting them into analysis (4,2).

Quality Assurance/Quality Control

Quality Assurance (QA) and Quality Control (QC) are the essential components of sampling of environmental samples for pesticides residue analysis. The laboratories are suppose to follow a system as per the ISO-17025 (or NABL guidelines) that includes essential components for sampling, preservation, storage, transportation and handover the representative samples to lab till the proper storage and recording of submission details are done. QA refers to all of the actions, procedures, checks and decisions undertaken to ensure the representativeness and integrity of samples and accuracy and reliability of analytical results. QC comprises those actions that monitor and measure the effectiveness of QA procedures with respect to defined objectives (1,2).

Therefore in order to achieve the test results in the environmental field an extremely careful approach is needed. Right from the planning of the sampling programme, preparation for sampling, sampling, preservation of sample, store and transport of sample, handling of samples in laboratory, processing of samples, analysis through

advance analytical instrumentation techniques, calculation, compilation and reporting of results. Each and every step is equally important. At every stage QA/QC must be ensured as the reported results would be used for taking vital decisions.

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Demo of Environmental sampling techniques

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Online demonstration through Video will organized

Sampling, Transportation and Storage of Samples

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Sample is a representative portion of material small enough in volume to be transported conveniently and handled in a laboratory. The relative proportions or concentrations of all pertinent components will be the same.

Sampling is a very important activity and should be done ina randomized way following international guidelines, e.g.European CommissionDirective 2002/63/EC or appropriate to ISO Standards related to the commodity of concern.

The representative samples of a small part of the environment in which we are interested should be collected and analyze these to provide information about the composition of the area. For example, it is impossible to analyze all the water in a lake, so portions of the water must be collected and analyzed to determine the true concentrations of materials in the lake. Similarly, to study contamination around a leaking underground gasoline tank, numerous soil samples are needed to map the extent of the pollution.

It has to be kept in mind that only a small amount of sample (a few grams or millilitres) can be collected from a vast heterogeneous area. The basic objective is that the samples collected must actually represent the environment as accurately as possible. Major decisions are based on the results of the analyses. The steps involved in environmental sampling are:

- Development of a sampling plan, including where and when samples will be collected and the number of samples required.
- Collection of the samples.
- Preservation of samples during transportation and storage to maintain their integrity.

The sampling plan:

Sampling is done for monitoring purposes, as well as for research. Data may be collected to monitor air and water effluents or to characterize pollutant levels in environmental media (air, water, soil, biota). The objectives may be to comply with regulatory requirements, to identify long and short term trends, to detect accidental releases, or to develop a database or inventory of pollutant levels. Research may

involve studying the fate and transport of pollutants or identifying pollutant exposures for humans and animals. It is important to design these studies scientifically so that they are cost-effective and generate statistically significant information.

Sampling has to be carried out only after proper planning. If not correctly, all the efforts of collection, analysis and data interpretation will go waste. Precautions have to be taken to avoid the introduction of bias or error.

Criteria like the type of sample to be collected (water, air or soil etc.), the size of the sample to be collected, its homogeneity, the potential for the introduction of bias and errors, acceptance and rejection criteria, contamination from extraneous sources, tolerance limits, compositional standards should always be taken into account.

While there are mathematical formulas to determine how much sample is needed, depending on the variation in the composition of the sample e.g. different particles and the size of these particles, the parameters needed to use these equations are not often available when environmental samples are considered. For example: to determine the number of portions of soil required to fully characterize a landfill, the number of samples to be collected and analyzed would be more because of the non-homogeneity of the soil in the landfill.

Spatial and Temporal Variability

A single sample would adequately represent the actual situationIf an environmental domain is completely homogeneous. However, such a situation is hardly encountered in real life, as the environment is highly heterogeneous. Iy has also to be seen whether the system is *static* or *dynamic*. A system which doesn't change much with time is called a static system. A simple sampling plan incorporating the collection of samples from the designated area in a preformed grid across the sampling area would reflect all the non-homogeneity of the system. e.g. If a field is to be tested for a long-lived pesticide in the soil, that could be considered to be a relatively static system.

If the contents of the system changes with time then it is called a dynamic system. Most of the regions in nature are dynamic to some extent and show both spatial and temporal variation. The concentration of constituents in the river, a waste effluent stream or air sample in and around the factory may vary widely over a period of minutes, days, or hours. When this is to be characterized, sampling must be done at different times to collect a representative sample. In such a case, many samples of a constant volume need to be collected at random times or on a regular schedule and composted to get a true representative sample. For instance, if samples are taken at

random, these random times should include all periods of time including weekends and nights, as well as business hours.

In air sampling, the concentration of VOCs will vary from area to area within a small locality and also change with the time of day. Concentrations of compounds from automobile exhaust are generally higher during peak traffic hours in urban areas. As a result, to get a true representative sample to judge the air quality in an area, samples have to be taken or measurements made at different locations and at different times of the day. Even changes due to seasonal and weather factors have to be taken into consideration.

When the sample is collected from a large environmental domain, it can be conceptualized as a point in time and space. Space units, S_1, S_2... denote sites, cities, even countries. Specific sampling locations are located within each space unit, described as a three-dimensional space, using x, y, and z coordinates. So, measurements may be taken at each location at different points in time, and at different locations at the same time. For example, if ozone levels in an urban areaare monitored, where S_1 and S_2 denote two cities on opposite sides of a river. Within the city S_1, several locations (L_{111}, L_{112}, ...) are chosen and measurements are taken at two different vertical distances from the ground (V_{11} and V_{12}). Time periods T_1, T_2 might represent different seasons of the year. Within each season, t_{11}, t_{12}... would represent daily or weekly averages.

Development of the sampling plan

A sampling plan is a detailed outline of which what sample will be taken at what times, in what manner, and by whom. The plan is different for different purposes. e.g. if the aim is to study the heavy metals in a river, then the actual purpose has to be taken into account. The sampling plan for the metals in aquatic flora and fauna will be different from that of the metal contents in the river water or sediments. Some of the major steps involved in the development of a successful study are as follows:

- The goal of the study is outlined and the hypothesis to be tested is decided upon along with the data generated to obtain statistically significant information.
- The environmental population or area of interest is outlined.
- If air samples are to be taken, information about the weather patterns should be obtained along with the information about the physical environment.
- A thorough literature search of the area and all the available data from the previous studies may provide useful information about the trends and variability of the data in a similar field.

- If no previous data is available, then a pilot may be carried out to get preliminary information on which the main study will be based.
- The detailed procedures to be used for the measurements have to be outlined which will affect the way samples are collected and handled.
- A sampling design has to be formulated as to how many samples are to be collected and area to be covered in the study and the time frame of the study.
- Based on the project objectives, the frequency of samples to be taken, both in time and space is decided. e.g. whether the sampling is grab sampling over different time periods, or integrated samples (over what time period) etc.
- The quality of each of the processes involved in the study: sampling, laboratory analysis, contamination control, etc. has to be insured by proper planning and documentation.
- Uncertainty of the measurements is a must for all the data once the sampling and analysis is complete.
- Proper statistical evaluation of the data has to be carried out once the data is acquired.
- Proper evaluation of the outcome of the project needs to be done to find out whether the objectives have been achieved. If not, the additional work has to planned and executed.

Sampling Strategies

While deciding the strategy of sampling like the number of samples, collection time and the sampling location are the key factors. Some of the factors in determining a sampling strategy are:

- The study objectives: Different objectives require different sampling strategies. For example, if the objective is to measure the total release of heavy metals into a river by an industry, a 24 hour integrated sample may be taken. However, if the goal is to monitor for accidental releases, then sampling and analysis may have to be done almost continuously.
- The pattern and variability of environmental contamination decides the number of samples to be collected at different time intervals at different locations. If traffic patterns and meteorological conditions vary then pollutant levels in the air can vary significantly. In such cases a larger number of samples need to be analyzed.
- More the number of samples analyzed, higher the precision and accuracy. Based on the cost of the project, time and resources an effective sampling plan need to be worked out.
- A well-designed strategy is needed to obtain the maximum amount of information from the number of samples. The strategy may be a statistical or non-statistical one. Many other factors such as convenience, site accessibility, limitation of

sampling equipment and regulatory requirements also often play important roles in developing a sampling plan.

Various approaches are applied to sampling viz. systematic, random, judgmental (non-statistical), stratified, and haphazard and they may be applied alone or in combination with others. A statistical approach is taken for increasing the accuracy and decreasing the bias. For example in a factory discharging effluents in a flowing water body, naturally,concentration of the pollutants present in the wastewater outfall will be the maximum near the discharge point. Dividing the water surface into grid blocks and sampling in a regular pattern from all the blocks will be **systematic sampling** plan. Sampling from a few of the grid blocks in a random manner is called **random sampling** while concentrating on the area around the outfall is **judgmental sampling**. **Haphazard sampling** is just taking a few samples at locations chosen by the sampling person. A Sampling at real-time measurements all the time is termed as **continuous monitoring** which eliminates the errors due to time factor and reveals a near true situation of the pollution in that area. When a system contains several distinctly different areas, these may be sampled separately, in a **stratified sampling** scheme. The target population is divided into different regions or strata. The strata are selected so that they do not overlap each other. Random sampling is done within each stratum. For example, in a pond or a lagoon where oily waste floats over water and sediment settle to the bottom, the strata can be selected as a function of depth, and random sampling can be done within each stratum.

Types of samples:

Grab sample: A grab sample is a discrete sample which is collected at a specific location at a certain point in time. If the environmental medium varies spatially or temporally, then a single grab sample is not representative and more samples need to be collected.

Composite sample: A composite sample is made by thoroughly mixing several grab samples. The whole composite may be measured or random samples from the composites may be withdrawn and measured.

A composite sample may be made up of samples taken at different locations, or at different points in time. Composite samples represent an average of several measurements and no information about the variability among the original samples is obtained. A composite of samples which all contain about the same concentration of the analyte can give a result which is not different from that obtained with a composite made up of samples containing both much higher and much lower concentrations. During compositing, information about the variability, patterns, and trends is lost.

When these factors are not critical, compositing can be quite effective. When the sampling medium is very heterogeneous, a composite sample is more representative than a single grab sample.

Composite samples may be used to reduce the analytical cost by reducing the number of samples. A composite of several separate samples may be analyzed and if the pollutant of interest is detected, then the individual samples may be analyzed individually. This approach can be useful for screening many samples.

Sampling:

Sample collection is one of the most important steps in arriving at results very close to the real situation. Faulty sample collection may result in problems which cannot be undone by even a perfect analytical procedure while a good sampling plan will ensure that the samples obtained, when averaged, closely represent the bulk composition of the environment being measured. In addition to this care should be taken so that the samplesare collected and handled in a way that their chemical composition does not change by the time it is analyzed. Finally, the sampling must be done with the requirements of the analytical method in mind.

The sample size has to be adequate for the analysis and it varies from sample to sample and analyte to analyte. If the sample size is too small and the concentration of the analyte of interest is very less, the result may be below the detection limit of most of the analytical techniques. It is, therefore, desirable that the sample is sufficient to meet the minimum requirement of the analyst with an amount left for the reanalysis of the same analyte, if needed.

The most common environmental samples are air, water, soil, biological materials, and wastes (liquids, solids or sludges). Each type of sample matrix needs a different sampling technique, although the basic concepts are the same in each case. Before planning a sampling, background information about the site should be gathered especially about the sources of the pollutants being investigated.In the case of air sampling pollution sources in the vicinity, such as industries and traffic should be kept in mind. While sampling from a hazardous waste site the history of the site (e.g. types of wastes being dumped in the past) may be helpful in finding the location of maximum contamination. Various physical factors such as soil type, ground and surface water flows significantly affect the migration and distribution of pollutants in a contaminated site and these should be taken into account while planning the sampling.Physical factors viz. Similarly, for air sampling wind direction, temperature, and relative humidity should be considered before planning.

Samples in the Laboratory

Once the samples reach the laboratory, they have to reduce in size. This is done with an aim to take a representative sample. In the case of soil samples, they have to be air-dried ground and sieved to get a homogeneous sample. The bulk sample need to be put in a pile, quartered, the opposite quarters selected and the other two discarded. This process is repeated till a small workable sample size is obtained.

Water sampling:

Types of water samples can be ground water, precipitation (rain or snow), surface water (lakes, river, runoff, etc.), ice or glacial melt, saline water, estuarian water and brines, waste water (domestic, landfill leachates, mine runoff, etc.), industrial process water and drinking water.The composition of stagnant water varies with the seasons and also with ambient temperatures. In rivers, lakes and oceans the concentration of pollutants varies with depth and may also depend on the distance from the shore.

Many different types of manual and automatic samplers are commercially available. They are designed to collect grab samples or composite samples. Particular attention is given to material of construction of the sampler. Stainless steel or Teflon are preferred because of their inert nature.

Collection of biological samples:

Pollutants in various environmental compartments viz. water, soil and air finally find their way to accumulate in plant or animal tissues. Analysis of these samples shows the extent of pollution in that particular area. Many of the organic pollutants may get metabolised in the biological tissues and one may not find the parent compound but their metabolite/s. Other pollutants like metals are very stable and may remain there for long periods of time. A study of fish or other aquatic organisms from a water body indicates the extent of water contamination. The results not only indicate the quality of water but also the suitability of the fish for human consumption.Biological samples should be placed in suitable containers and chilled immediately to prevent any damage to the internal tissues. These should be transported to laboratory in the same condition and at the earliest.

Collection of soil samples:

Soil samples can be collected from the surface and from various depths using the appropriate digging and collection tools. Various tools are readily available for the purpose of soil collection viz. scoops, shovels, tube samplers etc. Once collected, they

can be transported to laboratory in high density polythene bags or bottles made of glass or high density plastic. If volatile organics or microbiological tests have to carried out in the samples, the samples need to be kept under cold conditions during transportation and storage.

Preservation of samples:

The collected samples are the representatives of the environmental compartments. Although the composition of the samples from the field to laboratory may not be exactly the same but proper preservation, transportation and storage helps in reducing the errors to a minimum extent. Common physical processes which may degrade a sample are volatilization, diffusion, and adsorption. Possible chemical changes include photochemical reaction, oxidation and microbial degradation.

The steps commonly taken to minimize sample degradation are the use of proper containers, temperature control, addition of chemical preservatives, and observance of the recommended maximum sample holding time. The holding time depends upon the analyte of interest and the matrix under considerations. If the holding time is unknown, one can make up a spiked sample, or store an analyzed actual sample, and analyze it at fixed intervals to determine the optimum holding time.

Volatilization

Analytes with high vapor pressures, such as volatile organic compounds and dissolved gases, such as HCN, SO_2, will readily escape from the sample by evaporation. In order to minimize volatilization and the interaction between the water and the vapor phase above, the sample containers are filled to the brim, so that there is no head space. The samples are usually held at 4^oC, on ice, to lower the vapor pressure.

Choice of Proper Containers

The surface of the sample container may interact with the analyte. For example, metals can adsorb irreversibly on glass surfaces, so plastic containers are often chosen for water samples to be analyzed for their metal content. These samples are also acidified with HNO_3 to help keep the metal ions in solution.

To prevent plasticizers, such as phthalate esters leaching from plastic containers into the sample, it is recommended to collect such samples in glass containers. Bottle caps should have Teflon liners to preclude contamination from the plastic caps. Many organic compounds tend to degrade under sunlight and therefore, amber coloured

glass bottles are recommended for sample collected meant for the analysis of organics e.g PAHs, pesticides etc.

Transport:

Samples must be transported under appropriate conditions to the laboratory in clean containers and robust packaging. Polythene or polypropylene bags, ventilated if appropriate, are acceptable for most samples but low-permeability bags (e.g. nylon film) should be used for samples to be analyzed for residues of fumigants. Samples of commodities pre-packed for retail sale should not be removed from their packaging before transport. Very fragile or perishable products (e.g. ripe raspberries) may have to be frozen to avoid spoilage and then transported in “dry ice” or similar, to avoid thawing in transit. Samples that are frozen at the time of collection must be transported without thawing. Samples that may be damaged by chilling (e.g. bananas) must be protected from both high and low temperatures. Rapid transport to the laboratory, preferably within one day, is essential for samples of most fresh products.

Storage:

Laboratory samples which are not analyzed immediately should be stored under conditions that minimize decay. Fresh products should be stored in the refrigerator, but typically no longer than 5 days. Dried products may be stored at room temperature, but if storage time is expected to exceed two weeks, they should be sub-sampled and stored in the freezer.

SAMPLING DETAILS TO BE RECORDED:

Details of every sample should be recorded preferably using tags or labels over the container/bottle. It should include:

- Sample details (Type/nature)
- Date of Sampling
- Time of Sampling
- Sampling Location
- Sample Collector
- Temperature of Sample
- Any other details
- Sufficient space should also be provided for further transfer entries etc.

APPARATUS AND CONSUMABLES FOR SAMPLING

- Proper sampling tools suitable for the type and source of sample
- Proper sample containers/bags
- Gloves
- Sample labels
- Sampling log book, pen, marking pen

- Paper towels
- Insulated /ice box for storage in cold condition

Further Reading:

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Modern Analytical techniques for the samples preparation of organic pollutants (SPME/SPE/DLLME)

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In the past 30 years, sample preparation/pre-treatment prior to chromatographic analysis has risen from near-obscurity to the prominent place it now holds in most studies on the trace-level determination of organic micro-contaminants in real-life samples. Traditionally, sample preparation is stated to be necessary for several reasons:

- Improvement of the chromatographic behaviour of the analyte(s),
- Improvement of detectability of the analyte(s), or
- Isolation of the analyte(s) from the matrix

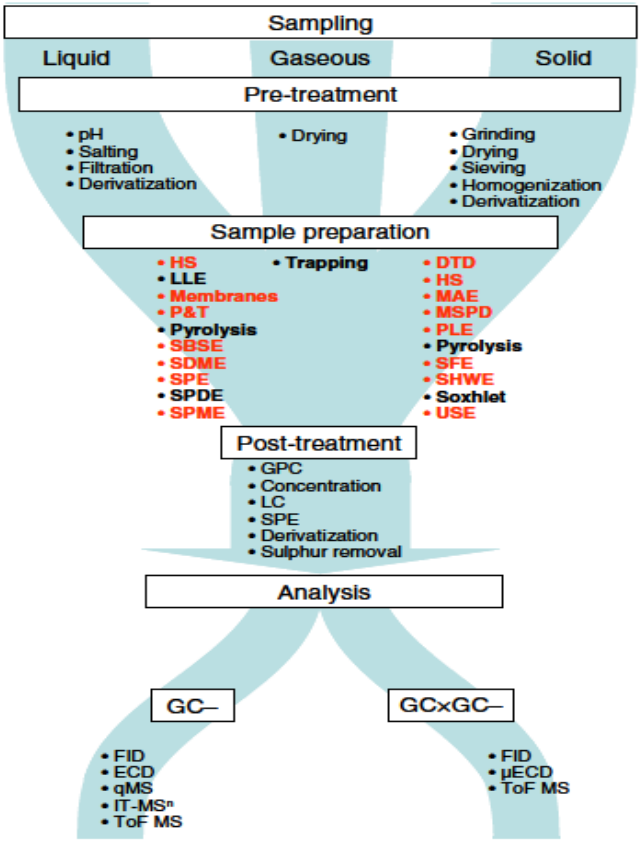
Today, the first aim has become relatively unimportant because of both the quality of column packings in gas (GC) as well as column-liquid (LC) chromatography and the essential superfluousness of derivatizing or labelling polar analytes to allow their determination by means of GC. The other two aims, viz. improved detectability and efficient separation from interfering sample constituents, are, however; as important as they were several decades ago. Over the years, it has increasingly been realized that, in many cases, sample preparation is the most time-consuming, tedious and error-prone step of the total analytical procedure. In addition, sample preparation often cannot easily be coupled on-line (or at-line) with the subsequent instrumental separation-plus-detection step, thereby making automation of sample preparation plus GC analysis essentially impossible.

Over the years, many groups of workers have attempted to improve the situation by designing new sample-preparation techniques (somewhat loosely called modern sample-preparation methods by most authors) to replace traditional methods such as Soxhlet, liquid–liquid (LLE) and ambient-pressure solid–liquid extraction—where one should immediately add that the former two methods are still widely used today, specifically in routine applications and, in the case of Soxhlet extraction, for reference purposes. The modern sample preparation techniques range from highly selective methods to be used for one, or a few, target analyte(s) of special interest to wide-ranging, and usually rather non selective procedures primarily meant for screening purposes, i.e., for target analytes as well as unknowns. Many methods can be made part of on-line (and, thus, automatable) systems, while others typically are off-line procedures. To enable their implementation, suitable sorbents, chemicals,

membranes, low-dead-volume connections, cartridges, mini-columns, disks, etc., have been synthesized and/or designed and, whenever required, instrumentation and ancillary equipment was constructed and, frequently, commercialized. Over the years, a variety of applications for widely different analyte/ matrix combinations have been published to demonstrate the practicality of the various approaches. Attention has been devoted, e.g., to designing integrated analytical systems, to miniaturization and to adequately matching the sample preparation and instrumental-analysis time. The main aims were, and still are, to increase sample throughput, improve the overall quality of the sample-preparation procedures, and decrease the required sample sizes and/or the use of organic solvents and sorbents, and the amount of waste.

One more aspect of interest should be mentioned here, that of improving detection limits. In the past ten to fifteen years, there has been an increasing, and fully justified, emphasis on the proper identification and/or identity confirmation of all analytes of interest in each sample. As a consequence, quadrupole- or ion-trap-based mass spectrometric (MS) detection is the state of-the-art approach today for a large majority of all challenging analytical procedures. The overriding importance of MS detection will readily become apparent from the many tables included in the Applications section of this review. Even element-selective detection only plays a modest role today. Its most prominent application areas are the trace-level determination of organochlorine (and -bromine) micro-contaminants by GC with electron-capture detection, and the selective screening of organo-sulphur compounds by GC with S-based chemiluminescence detection. Today, a wide variety of analytical methods is available for the GC determination of organic micro-contaminants in sample types such as air, water and other liquid samples, soils and sediments, fish and food, and biota. In the present review, we focus on the sample-preparation step—with examples primarily relating to liquid and solid samples—and, more specifically, on the characteristics of the modern techniques, i.e., those introduced in the past twenty or so years. These are marked in grey (electronic version in red) in the figure. All acronyms used in this figure and throughout the review are summarized in the glossary at the end of this review article. In the sub-sections, each of the separate techniques will be briefly described, and a number of selected applications, strategies and on-going developments will be given to illustrate the merits and demerits of each of these. For each technique, a number of recent reviews and/or other general reference sources will be given; in many cases, these have been used as the backbone of this chapter. Aspects such as spiking and recovery of analytes, and quantification (inclusive of validation and matrix effects) will not be discussed.

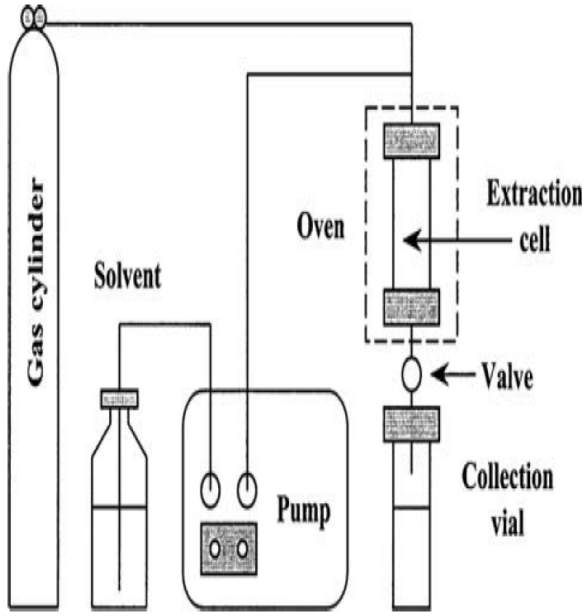
A typical schematic which displays most of the more important routes is



Sample Preparation Methods

1. Pressurized Liquid and Subcritical Hot-Water Extraction:

Pressurized liquid extraction (PLE) involves extraction with solvents at elevated pressures (up to ca. 20 MPa) and temperatures (up to ca. 200 C) without their critical point being reached, to achieve rapid and efficient extraction of trace-level analytes from a (semi-) solid matrix. Since its introduction in 1995, PLE, also known as accelerated solvent extraction (ASE) and pressurized fluid extraction (PFE), several reviews have been published and the technique has been shown to have significant advantages over competing techniques such as Soxhlet, Soxtec, and microwave assisted extraction (MAE) extraction: enhanced solubility and mass-transfer effects and the disruption of the surface equilibrium are the main beneficial causes. As a consequence, compared with Soxhlet extraction, both time and solvent consumption are dramatically reduced. Originally, the use of PLE mainly focused on the isolation of organic micro-contaminants from environmental matrices such as soil, sediment and sewage sludge. Today, the technique is also used for the analysis of, e.g., food and biological samples. Instead of an organic solvent, pure water can also be used for extraction. In that case, the technique is usually called subcritical hot-water (SHWE) or pressurized hot water (PHWE) extraction.



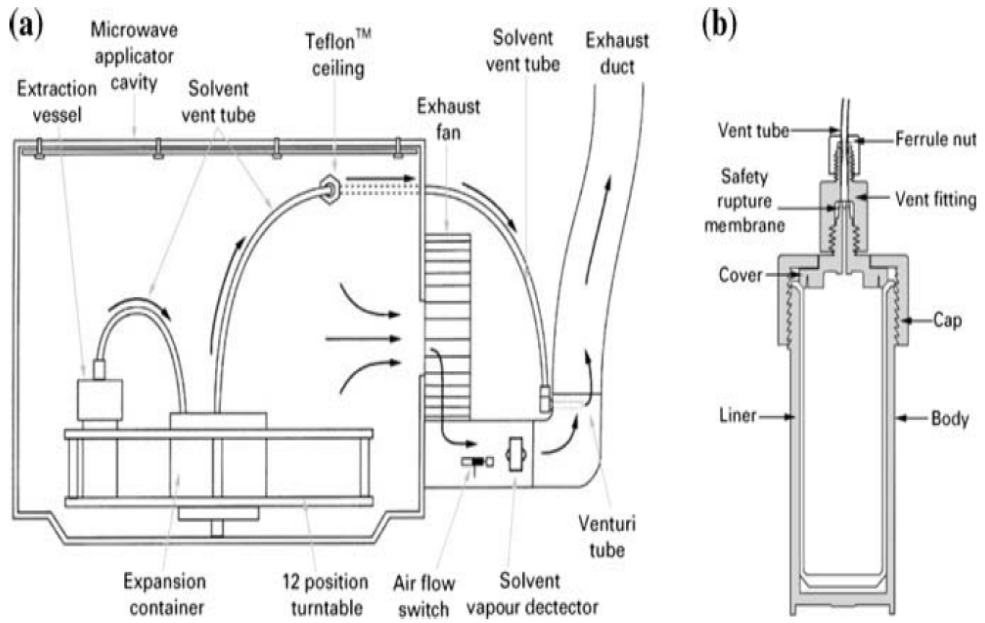
Schematic representation of a PLE system

In general, higher temperatures will cause an increase of the PLE efficiency due to enhanced sample wetting, better penetration of the extraction solvent, and higher diffusion and desorption rates of the analytes from the matrix to the solvent. They are therefore recommended provided there are no limitations associated with thermo labile analytes and/or matrices. To quote an example, a temperature of 100 C is often selected as 'default value' and used for the PLE of POPs (persistent organic pollutants) from a variety of matrices with different solvents, while mixtures containing toluene often require temperatures close to 200 C to provide maximum recoveries.

2. Microwave-Assisted Extraction:

Today, MAE is widely recognized as a versatile extraction technique, especially for solid samples. MAE utilizes electromagnetic radiation to desorb analytes from their matrices. The microwave region is considered to exist at frequencies of 300 MHz to 100 GHz. Although the whole of this region is potentially available for use, all (domestic and scientific) ovens operate at 2.45 GHz only.

The main advantages of MAE are the usually high extraction rates due to the very rapid heating and the elevated temperatures, and the ease of instrument operation. A drawback is that the heating is limited to the dielectric constant of the sample/solvent. The primary mechanisms for energy absorption in MAE are ionic conductance and rotation of dipoles. Ionic-conductance heating is due to the electrophoretic migration of ions when a microwave field is applied. The resistance of the matter to this flow will generate heat as a consequence of friction. Dipolar molecules couple electro statically to the microwave-induced electric field and tend to align themselves with it. Since the microwave field is alternating in time, the dipoles will attempt to realign as the field reverses and so are in a constant state of oscillation at the microwave frequency. Frictional forces cause heat to be developed due to the motion of the dipoles.



Schematic diagram of a) A Closed vessel MAE system b) A standard lined extraction vessel

In MAE, sample and organic solvent are subjected to radiation from a magnetron. There is a high cost differential between microwave ovens for domestic use and for MAE, which sometimes precludes the purchase of a dedicated MAE system. However, for safety reasons (explosions in the presence of an organic solvent), it is strongly recommended to use only dedicated systems. Although the application of several brands and models is reported in the literature, there is a tendency for the models of CEM and Milestone.

3. **Ultrasound-Assisted Extraction**:

In ultrasound-assisted extraction (USE), acoustic vibrations with frequencies above 20 kHz are applied to extract analytes from permeable (semi-)solid matrices. The top end of the frequency range is limited only by the ability to generate the signals; frequencies in the GHz range have been used in some applications. Sound waves are intrinsically different from electromagnetic waves: while the latter can pass through vacuum, sound waves must travel in matter, as they involve expansion and compression cycles travelling through a medium. In a liquid, the expansion cycle produces negative pressure and bubbles or cavities are formed. When a bubble can no longer efficiently absorb the energy from the ultrasound, it implodes. The whole process, known as ‘cavitation’, takes place within about 400 µs. Rapid adiabatic compression of gases in the cavities produces extremely high temperatures and pressures, estimated to be about 5,000 C and roughly 100 MPa, respectively. The high temperatures and pressures cause the formation of free radicals and other compounds; for example, the

sonication of pure water causes thermal dissociation into hydrogen atoms and OH radicals, the latter forming hydrogen peroxide by recombination.
There are two common devices for ultrasound application, bath and probe systems. The baths are more widely used, but have two disadvantages, which adversely affect experimental precision, viz. a lack of uniformity of the distribution of ultrasound energy (only a small fraction of the total liquid volume in the immediate vicinity of the source will experience cavitation) and a decline of power over time. The probes have the advantage over baths that they focus their energy on a localized sample zone and, thus, provide more efficient cavitation in the liquid. Most USE applications have been developed using a bath or a probe. Dynamic systems (DUSE) have been used in a few cases only, even though this approach will speed up the USE process considerably. There are two DUSE approaches, open and closed systems.

4. Supercritical Fluid Extraction:

One area that stimulated an interest in enhanced fluid extractions was supercritical fluid extraction (SFE). This is a long established method, which has been used industrially for many years. However, it was not until an interest was shown in supercritical fluids as chromatographic media that it started to be seriously studied as an extraction technique on an analytical scale. It has since been the subject of numerous books and reviews.

Almost all SFE employs carbon dioxide (critical point, 30.9 C, 73.8 bar) as the supercritical fluid: it is an almost ideal solvent since it combines low viscosity and high analyte diffusivities with a high volatility (which makes analyte recovery very simple and provides solvent-free concentrates), and is inexpensive and environmentally friendly. An important drawback of CO2 is its nonpolar character. In order to widen the application range of the technique to include more polar analytes, the preferred route is to employ polar modifiers such as methanol, ethanol, acetone and acetonitrile (1–10% addition, preferably by means of a separate modifier pump). In addition to a modifier pump, the basic components of an SFE system are: a supply of high purity carbon dioxide; a CO2 pump; an oven for the extraction vessel; a pressure outlet or restrictor; and a suitable collection vessel for recovery of the extracted analytes. Sample collection can be performed by purging the extract through a solvent or over a suitable adsorbent, such as, Florisil.

5. Matrix Solid-Phase Dispersion:

The analysis of (semi-) solid environmental, food or biological, sometimes fat-containing matrices is a challenging problem, with rapid and efficient analyte isolation and subsequent purification being of key interest. In 1989, Barker et al. introduced

matrix solid-phase dispersion (MSPD) and the technique has since then been discussed in several reviews. MSPD involves the direct mechanical blending (for solid samples) or mixing (for semisolid and liquid samples) with, usually, an alkyl-bonded silica SPE sorbent but, occasionally, also plain silica, Florisil or sand. The added abrasive promotes the disruption of the gross architecture of the sample while, with a bonded silica, sample constituents will dissolve and disperse into the bonded phase, causing a complete disruption of the sample and its dispersion over the surface. When blending or mixing is complete, the homogenized mixture is packed into an empty column or cartridge (with, usually, frits, filters or plugs at both top and bottom). Obviously, there is one main difference here between MSPD and SPE: with the former technique, the sample is distributed throughout the column and not only retained in the first few millimetres. Elution with, preferably, a limited volume of solvent is the final step of the remarkably simple procedure.

The elution solvent should effect an efficient desorption of the target analytes while the bulk of the remaining matrix components should be retained. In the literature, a wide variety of solvents has been tested, ranging from hexane and toluene, via dichloromethane and ethyl acetate, to alcohols and water at elevated temperatures. Not surprisingly, pesticides are usually eluted with low- or medium-polar solvents, and drugs and naturally occurring compounds with more polar ones. Generally speaking, the nature of the preferred sorbent/solvent combination is mainly determined by the polarity of the target analytes and the type of sample matrix. Keeping this common-sense consideration in mind will facilitate MSPD optimization.

6. Direct Thermal Desorption:

Thermal desorption (TD) is a valuable alternative to headspace techniques for the isolation of volatile compounds from non-volatile solid, semi-solid and, occasionally, liquid matrices, and a wide variety of applications has been reported in the literature. Although TD is not really a new technique, fully automated systems are only in use for slightly over 10 years. One of the first examples was the use of automated thermal desorption (ATD) for the determination of volatile constituents of plants and food. Typically, a 1–40 mg sample is placed in a desorption cartridge between two glass-wool plugs. By heating the cartridge for a pre-set time, the volatiles are desorbed and, next, adsorbed on a cold Tenax trap. Heating of the trap effects rapid transfer of the analytes to the GC for further analysis. Similarly, TD–GC– MS can be used as a screening method, e.g., for chlorinated hydrocarbon contamination in soil. In this case, a dual-tube system was used to enable focusing of the analytes on a Tenax-plus carbon trap prior to their release and transfer to the GC system. Total analysis including the sample preparation, required less than 1 h. TD is also used to study the relatively low-molecular-mass components present in (oil-containing) rocks. Pyrolysis

techniques are used to study the very-high-molecular mass structures in such samples which are not directly amenable to GC. An inexpensive and user-friendly system for multi-step TD–pyrolysis–GC for use in geochemical analysis was designed by van Lieshout et al. Thermal treatment was performed inside a PTV injector which served as both the TD unit and the pyrolyser system. Sample amounts ranging from sub-mg amounts up to 2 g were weighed directly into the liner of the injector. The system was also used for polymer characterization.

TD is being increasingly used for the analysis of aerosols and, also in this area of application, primarily in order to replace time-consuming procedures involving solvent extraction (and evaporation) by more direct approaches in which (parts of) aerosol-loaded filter material is packed into a GC injector liner and directly subjected to thermal desorption plus instrumental analysis . The main advantages are reduced sample handling and improved analyte detectability (9– 500 times better LODs than solvent extraction), while there is no need to modify the GC–MS set-up.

6. Solid-Phase Extraction:

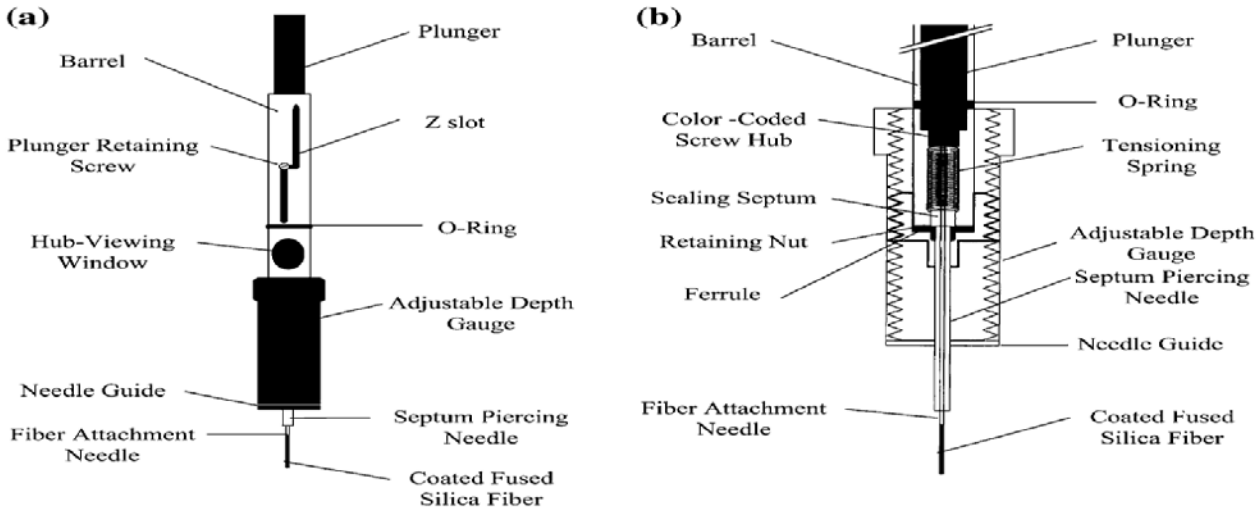
In the late 1970s, SPE was introduced for the pre-treatment of aqueous samples. Since that time, off-line and, specifically, on-line trace enrichment and clean-up by means of SPE using pre columns or (disposable) cartridges has become a very popular—probably the most popular—column-switching technique in LC. Most techniques and much of the hardware used today for off-line SPE-GC and on-line SPE–GC were adapted from the corresponding LC techniques. In the 1990s, semi- and fully automated systems were designed for both chromatographic techniques, and scores of off-line, at-line and on-line applications were reported. Consequently, many of the more informative reviews were published in that period—with environmental applications being the main field of interest for GC-based studies.

SPE cartridges have dimensions of, typically, 10–20 mm length x 1–4.6 mm ID. In most instances the cartridges are packed with 10–30 lm sorbents such as C18- or C8-bonded silica or styrene– divinyl benzene (SDB) copolymer. These are essentially non-selective sorbents because for many applications the SPE step should primarily guarantee the enrichment of analytes covering a wide range of polarities, with the subsequent chromatographic separation (plus detection) step ensuring the proper recognition of the individual compounds. Since separation-plus-detection is much more powerful in GC than in LC analysis, with the former technique the bonded silica's and the copolymer are virtually the only sorbents used in real-life applications. A typical set-up for SPE–GC is depicted in Fig. 14. After cartridge conditioning, a sample volume of, often, some 10 mL is loaded at a speed of several mL min-1 , the cartridge is cleaned with a few millilitres of water, and dried for some 20– 30 min with nitrogen at

ambient temperature. Next, the enriched analytes are desorbed with as little as 100 µL of an organic solvent—frequently ethyl or methyl acetate—and transferred on-line to the GC part of the system for further analysis. There is abundant experimental evidence that with, e.g., GC– MS, GC–NPD or GC–AED as instrumental analytical techniques, LODs of 5– 50 ng mL-1 can be obtained for a wide variety of micro-contaminants in 10-mL real-life samples.

8. Solid-Phase Micro-Extraction:

In 1990, solid-phase micro-extraction (SPME) was introduced by Arthur and Pawliszyn as an organic-solvent-free extraction technique. The theory and practice of the method have been examined in considerable detail and numerous applications have been reported and reviewed. Basically, the technique enables the trace enrichment of analytes by the exposure of a fused-silica fibre coated with an appropriate sorbent layer, for a selected time, to a gas or liquid sample, with the subsequent (rapid) desorption of the target analytes by heating the exposed fibre in the injection port of a GC. A number of fibre coatings, which offer a range of analytesolubilities and porosities, are commercially available. These include the highly popular non-polar polydimethylsiloxane (PDMS) and more polar coatings such as PDMS–divinylbenzene copolymers, poly acrylates and mixtures of carboxen (an inorganic adsorbent) and PDMS or divinylbenzene. Their mutually different physicochemical characteristics help to widen the application range of the technique. Fibre coatings are available in increasing thicknesses from 7–150 lm, which increases the partitioning ratio of the target analytes—and, hence, analyte detectability—but also increases equilibration times.



Commercial SPME device a) SPME fiber holder b) section view of SPME holder and fibre assembly

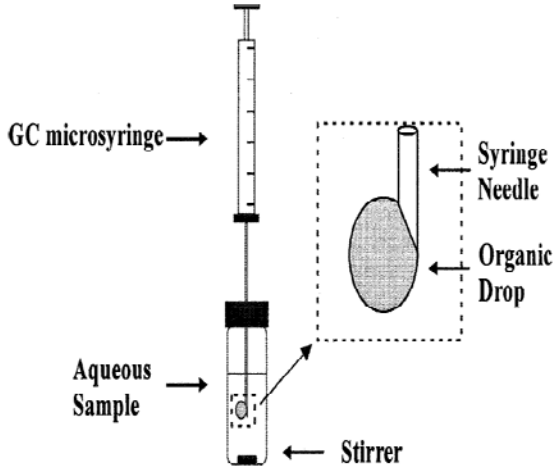
In a typical SPME experiment, the coated fibre is exposed directly immersed in, or to the headspace of, a small volume of liquid or sample extract, usually some 2–5 mL.The analytes partition into the stationary phase until plateau conditions are reached, which typically takes 2–60 min. The process can be aided by salting-out (addition of, e.g., 25% NaCl) and/or pH adjustment, sample agitation (to speed up analyte transport from the bulk of the solution to the vicinity of the fibre) and heating [232, 233]. Adverse matrix effects can be avoided by applying a standard addition procedure for quantification or, less frequently, using protective membranes to prevent adsorption of matrix components on the fibre. If selective detection, such as MS in the SIM mode or ECD, is used, LODs for both volatile and semi-volatile analytes typically are in the low-ng/mL, and sometimes in the ng/L, range. However, one should consider that SPME is an equilibrium technique. That is, although favourable analytes can be extracted essentially quantitatively, there are also many classes of compounds for which this is certainly not true actually, it is not unusual to find recoveries of less than 10% in the published literature. For such classes of compounds, conventional SPE can always provide better analyte detectability. Admittedly, non-equilibrium methods can also be used for SPME and also for SBSE and HS but this will decrease method sensitivity and will require highly precise timing procedures.

The SPME technique is marketed by Supelco (Bellefonte, PA, USA). Most reported applications are of the manual type. However, automated analysis can be performed by using systems commercialized by Varian and CTC. Recently, Pawliszyn and his group reported the automation of SPME on a 96-well plate format, which they claim to be a viable approach compatible with both GC and LC platforms.

9. Single-Drop Micro-Extraction:

In 1996, Liu and Dasgupta, and Jeannot and Cantwell introduced the concept of using a small drop for sample preparation, so-called single drop micro-extraction (SDME), which combines analyte extraction and preconcentration prior to instrumental analysis. Liu and Dasgupta reported a ‘drop in-drop’ configuration in which a 1.3 µL organic drop, suspended in a larger aqueous drop, extracts the analyte of interest. The system has the advantages of low consumption of organic solvent and the facility of automated backwash. Jeannot and Cantwell introduced a technique where an 8 µL drop of organic solvent containing an internal standard is left suspended at the end of a PTFE rod immersed in a stirred aqueous sample solution. After sampling, the rod is withdrawn from the solution and, with the help of a micro-syringe; an aliquot of the drop is injected into a GC system. As a more convenient alternative, microextraction

can be performed by suspending a 1 µL drop directly from the tip of a micro syringe needle immersed in a stirred aqueous sample. After extraction, the micro drop is retracted back into the needle and, next, transferred to the GC. Since droplet instability at high stirring speeds can cause problems, while such high speeds are usually beneficial because they enhance extraction, the use of a modified tip design was recommended in recent work.



The similarity of SDME and SPME operations suggests that auto samplers that can be used for SPME should also work with SDME. First results using a 2 µL drop of hexadecane for BTX analysis using a CombiPALautosampler, and a standard 10 µL micro -syringe confirmed this supposition. A single magnet mixer was used to permit temperature controlled extractions while stirring the sample.

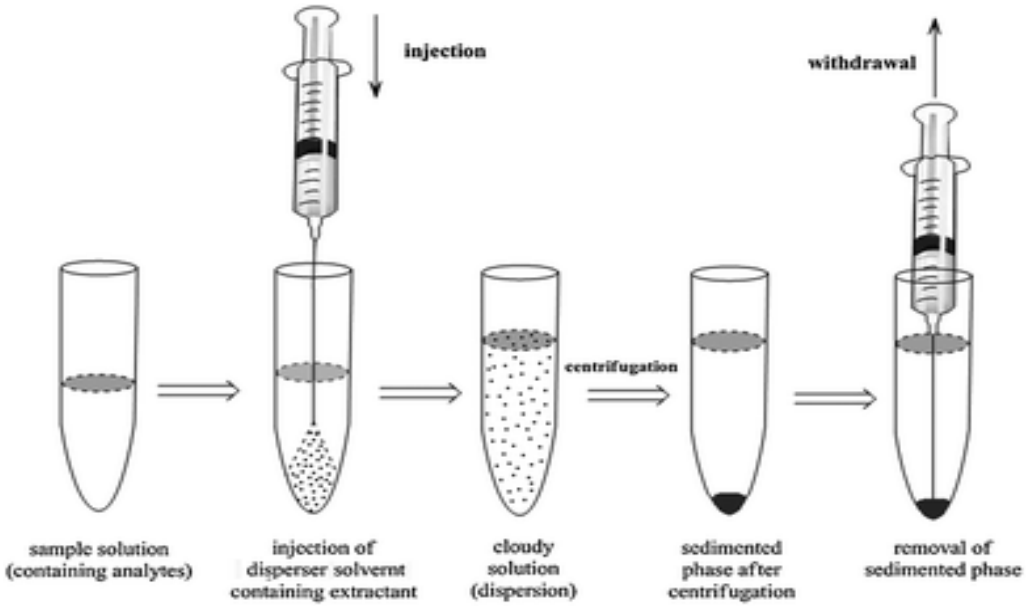
In an interesting study, HS-SDME and simultaneous derivatization were applied for the determination of acetone in human blood as a diabetes biomarker. A 1-mL blood sample was introduced in a headspace vial. Derivatization and extraction of acetone were performed by using 2 µL n-decane containing PFBHA, at an extraction temperature of 25 C and an extraction time of 4 min. Analyte recovery was 88% and the LOD for MS detection was 2nM. In another study, OPPs were determined in orange juice. 5% NaCl was added to 5 mL of orange juice for salting out the analytes of interest. SDME was performed by immersing the syringe needle in the sample, exposing a 1.6-lL drop of toluene during 15 min (stirring at 400 rpm). With analyte recoveries of 76–108%, the LODs for FPD detection were below 5µg/L. A third example shows that even SDME can be miniaturized. In so-called drop-to-drop solvent micro-extraction (DDSME), the extraction of methoxyacetophenone isomers from water was performed in a 100 µL vial containing one drop (7µL) of water. A 0.5 µL drop of toluene was exposed to the sample for 5-min extraction (stirring at 360 rpm and room temperature). The extractant was directly injected into a GC–MS system and LODs of 1 ng mL^{-1} were obtained for all isomers.

10. Dispersive liquid-liquid micro extraction:

The selective extraction of analytes is based on their different chemical and physical properties, including molecular weight, charge, solubility, polarity, and volatility. Numerous techniques exist for extracting the sample, each one appropriate for a given type of analyte or matrix. Despite the advanced separation and identification

Microextraction techniques available, food, water, samples still require a previous treatment. Conventional methods of sample preparation based on wet digestion are time-consuming, require great amounts of reagents, generate numerous residues, and may lead to a distortion of the results owing to both the impurities present in the reagents and the prolonged manipulation involved.

DLLME is an appropriate tool for the analysis of samples with a relatively simple matrix, such as water samples. However, interest and the number of applications in other fields such as food analysis have increased during the last few years. Owing to the interaction of the matrix components of this type of sample with organic solvents, it is more difficult to obtain a sedimented phase appropriate for injection in gas chromatography (GC) or liquid chromatography (LC). Thus, for food samples, dilution or pre-treatment of the sample, including an extraction and/or cleaning step, is necessary. A significant number of GC-based analytical procedures involving conventional DLLME have been proposed for the determination of volatile and semi volatile compounds in food samples. For species of low volatility or high polarity, as is the case of phenolic compounds, most authors use derivatization reagents compatible with the aqueous phase, such as acetic anhydride. The acetylation of bisphenol A and bisphenol B has also been performed in an organic phase, the acetonitrile sample extract, which is then used as the disperser solvent in the microextraction step. Derivatization and microextraction have also been performed in a single step for the determination of biogenic amines in beer, the chemical reaction occurring within the organic phase. The excess of isobutyl chloroformate, the derivatization reagent, was eliminated prior to GC injection to prevent degradation of the stationary phase material.



Although most DLLME studies have been applied to organic compounds, inorganic analytes have also been determined. The electro thermal atomic absorption spectroscopy technique uses micro amounts of sample for analysis. Thus, a good combination is achieved by coupling DLLME and electro thermal atomic absorption spectroscopy. In this approach, a chelating agent is added to the sample solution and then DLLME is performed.

As already mentioned, DLLME has been extensively applied to the determination of both organic and inorganic species in water samples. However, for other matrices such as foods and biological samples, the number of applications for inorganic analytes is relatively low owing to both the complexity of the matrix and interference effects. Additional pre-treatment steps are usually required in order to obtain a solution suitable for DLLME

Spiking of Standards in samples for recovery study using case history

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TO GET PROPER RECOVERY

Wrong or inaccurate measurements can lead to wrong decisions, which can have serious consequences, costing money and lives. The human and financial consequences of wrong decisions based on poor measurement being taken in matters as important as environmental changes and pollution are almost incalculable. It is important, therefore, to have reliable and accurate measurements, which are agreed and accepted, by the relevant authorities worldwide. Metrologists are therefore continuously involved in the development of new measurement techniques, instruments and procedure to satisfy ever-increasing demand for greater accuracy, increased reliability and rapidity of measurements.

Consultative committee for Amount of Substance (CCQM) is the committee responsible to provide apex traceability to the chemical measurements. It is responsible for the work in all the areas of chemical measurements / chemical metrology.

Chemical metrology is the science of measurement, embracing both experimental and theoretical determinations at any level of uncertainty in the field of chemical sciences. The unit accepted internationally (SI Unit) for all the chemical measurements is mole and its symbol is " mol".

Following methods / techniques are being used to measure any chemical parameter.

- **Volumetric**
- **Gravimetric**
- **Potentiometric**
- **Coulometric**
- **Voltametric**
- **Spectrometry**
- **Mass spectrometry**
- **Chromatography**
- **Chromatography Mass spectrometry**

Units of the measurements

Following units are commonly used in measurements

- **Percentage (%)**
- **Parts per million (ppm)**
- **Parts per billion (ppb)**
- **Parts per trillion (ppt)**

Of other parameters the units being used are:

- **PH: milivolt (mv)**
- **Conductivity: siemens(s)**
- **Resistivity : ohm ( )**

All the units could be correlated with the SI unit mole adopted for chemical measurement. “The **mole** is the amount of substance of a system which contains as many as elementary entities as there are atoms in 0.012 kilograms of Carbon-12”

The methods/ techniques used in measurements giving the values in SI units or its derived units are primary techniques. These measurements are directly traceable to international or national measurements system in case equipment used in measurement is calibrated and traceable to these measurement systems.

Traceability: Property of the result of a measurement or the value of a standard whereby it can be related to stated references, usually, national or international standards, through an unbroken chain of comparisons all having stated uncertainties. The concept is often expressed by the adjective Traceable and the unbroken chain of comparisons is called a Traceability chain.

The volumetric and gravimetric techniques are the primary techniques as they are directly traceable to the SI unit mass (kg). Similarly potentiometer, coulometric and voltametric techniques are also primary techniques as they are directly traceable to the SI unit current (A). The other properties namely pH, conductivity and resistivity are being measured electrically hence these are also the primary techniques and having the traceability.

The others techniques namely spectrometry, mass spectrometry, chromatography, chromatography-mass spectrometry etc. are relative techniques as they are not directly giving the values corresponding to the standards used in measurement.

To achieve the traceability all the measuring equipment should be validated by the use of certified reference materials (CRMs) and methods should also be validated by their use. The techniques are very sensitive techniques to measure the various characteristics in very wide analytical range and are being used in modern laboratories.

Reference standards used in certain laboratories report undoubtedly inaccurate results. In this way the proper preparation and storage of analytical standard solutions is of utmost importance. Incorrect standards will result in corresponding incorrect analytical data even though first class technique is thereafter employed and all laboratory instruments are in perfect operating condition.
Reference standards of organic compounds are subject to a wide variety of oxidation, hydrolysis, isomerization and polymerization reaction. Instability of organic standard is therefore, often a problem. Storage and use conditions and purity should be periodically checked.

Reference Materials:
A material or substance, one or more of whose property values are sufficiently homogeneous and well established to be used for calibration of an apparatus, the assessment of measurement methods or for assigning values to the materials.

Certified Reference Materials (CRMs)

A reference material, accompanied by a certificate, one or more of whose property values are certified by a procedure which established its traceability to accurate realization of the unit in which the property values are expressed and for which each certified value is accompanied by an uncertainty at a standard level of confidence.
In the present scenario of globalization of trade, the use of reference material (RM) and certified reference materials (CRMs) is essential for global acceptance of industrial produces and test and calibration report, these are being used for calibration of analytical equipment and validation of test methods to get precise, accurate and traceable values. These also provide traceability to the national and international measurement systems, Traceability is an essential aspect of quantity assurance (QA) if international acceptance of analytical data based on confidence and reliability is to be achieved. The use of CRMs is also a mandatory requirement for accreditation of the laboratories under ISO/IEC-17025.

National Metrology Institutes (NMI) had started production and certification of the CRMs for calibration of such type of techniques and validation of test methods .A large number of CRMs are required to meet the requirement of all type of testing and till recent very few organizations are producing it. The oldest CRM producer is National Institute of Standards and Technology (NIST) USA .In view of globalization of trade and implementation of common quality system, the demands of CRMs is enhanced tremendously. Therefore large numbers of NMI had started to produce CRMs.

Reference materials must be

- Characterized (certified value)
- Homogeneous
- Stable
- Certified, traceable reference materials Purchased from reputable sources
- Reference materials and items used for no other purpose than calibration.
- Check intermediate materials and items regularly against reference standards.
- Store reference items and materials carefully**.**

CRMs selection is based on

- Material of interest
- Analysis of interest
- Analysis concentration level
- Homogeneity
- Stability and self-life
- Quantity of CRMs
- Health and safety

In estimation of pollutants the ppb or subppb standards are required, which are prepared in following three steps

STANDARD

1. Stock solution from primary standard
2. Intermediate solution from stock solution of primary standard

3. Working standard proposed from intermediate solution

PRIMARY STANDARD: -

They are of analytical grade standard (reference/certified) of >99 % purity. Purities of standard are commonly greater than 99%and seldom less than 95%but may be lower in some cases .The percentage of purity must be known in order to apply a correction factor in weighing on the standard for subsequent dilution. Ifprimary standard is given 99%pure, weigh 20.00/0.99=20.2mg instead of weighing 20mg.Pure primary standards must be purchased from reputed manufacturer (List given separately) along with certificates consisting of their purity, self-life, storing condition, handling etc.

Toxicity levels and relative stabilities are important factors that dictate the method of handling and storing various standards. High toxic compounds (Low LD50 values) require special precaution such as wearing disposable rubber or plastic gloves and avoiding inhalation of vapors.

Primary standards are following type

1-Neat -pure material not dissolved in any solvent.

2-Single component solution: pure material quantitatively diluted into solvents, available in glass ample or in LDPE bottles.

3-Mixed component solution: pure materials quantitatively diluted into solvent with fixed concentration of each component. Available in glass ample for organic while inorganic are available in LDPE bottles.

STORAGE OF PRIMARY STANDARD: -

Solution in samples must be stored in fridge and allowed to come to room temperature before in further dilution while solids are either stored at room temperature in highly sealed condition if stable at room temperature as organic chlorine pesticide or in a refrigerator as in case of organophosphorus pesticide. The refrigerated material must be allowed to come to room temperature in the desiccators before use.

Secondary standard

From the name itself it is obvious that this is a standard which comes second. That's why the name is secondary

A secondary standard is a chemical or reagent which has certain properties such as

(a) It has less purity than primary standard

(b) Less stable and more reactive than primary standard

(c) But its solution remains stable for a long time

(d) Titrated against primary standard

Usually a chemical fit for being a standard chemical yet does not meet the requirements of a primary standard.

(A) Preparation of concentrated stock solution –

Secondary standards are liquid solutions of the primary standards. The final concentration of working standard will depend upon its use, eg

- pg range for GC equipped with ECD
- ng range for GC/TLC equipped with other detectors
- µg range for spectroscopy.

Crystalline standards are weighed on aluminum foil using electronic balance capable of weighing to at least 0.0001g. Transfer the standard into the volumetric flask through a small glass funnel. The foil and funnel being careful rinsed with solvent. Standards not crystalline should be weighed in small glass beaker are completely dissolved (observe carefully) in a small volume of solvent and transferred quantitatively by rinsing with the rest of the solvent through funnel into the volumetric flask. Liquid primary standards can alternatively be transferred to stoppered volumetric flask or drop bottle using glass dropper by weight. This closed dropping bottle technique is mandatory for high toxicity liquid compound.

It is difficult with any of these techniques to weigh exactly the predetermined amount to obtain all solutions of exact concentrations. Therefore it is to be done by either Dissolving the known amount and making up to known volume and recalculate the strength of solution.

Or

Dissolving known weight and make up the volume to known volume with solvent to get the desire concentration.

NOTE-shake well to get dissolve the crystal in solvent and left for overnight for complete dissolution.

(B)Preparation of intermediate concentration standards-

it is impractical and hazardous to prepare the final solution from the concentrated standards in one dilution or to prepare a original secondary (intermediate) standard at concentration low enough to allow only one subsequent dilution.

Separate solutions of each compound or a standard mixture can be prepared at this point .It is prepared by diluting the stock solution. It may require several dilutionsteps to getproper concentration micro liter stock solution not be collected in volumetric flask for preparing intermediate standards.

C) Working standard-

Wworking standard are generally made up on mixture, the actual combination being dependent upon the compounds of interest and the ability of the analytical method to resolve them. Care must be exercised that these mixtures donot contain compounds that may react with each other. Each working standard mixture should be made up at two or even three concentration each depending on the variation in the compound concentration in routine samples . No compound should be present in such concentration that when injected into the chromatography equipment the linear range of the detector will be violated.

DO AND DON`T

DO-

1) Always Use ‘A’ grade Calibrated Glass Wares (Pipette & Volumetric Flask) Preferably New One.

2) Use Balance With Capability to Weigh Minimum 0.0001g.

3) Be Sure About Dissolution Of Compound For Preparation Of Stock Solution.

4) Always put level on each flask.

5) Always mark on flask after delivering the solution.

6) Put all the standard solution prepared in fridge.
7) Always use separate pipettes for individual one for each dilution.
8)Always use rubber suction bulb.
9) Always use fresh bath of solvents preferably prechecked for the impurities

DON'T-

(1) Never use plastic containers or pipette.
(2) Never use micropipette.
(3) Never pipette by using mouth suction.
(4) Never use expired shelf life standard.
(5) Never use solvents that are already in use.

INTERNAL STANDARD-

An Internal standard is a known quality of a compound, different from analyte, generally not detected in sample that is added to the standard solutions. Signal from analyte is compared with signal from the internal standard to find out how much analyte is present.
Internal standards are especially useful for analysis in which the quantity of sample analyzed or the instrument response varies slightly from run to run for reasons that are difficult to control. For example, gas or liquid flow rates that vary by a few percent in chromatography experiment could change the detector response. Internal standards are widely used in chromatography because the small quantity of extracted sample solution injected into the chromatograph is not very reproducible in some experiments.
Care should be taken that amount of the internal standard is well above the limit pf quantitation but not so high as suppress the resolution of other analytes. Generally 1/3 of the working standard curve is added as IS in standard solution for eg.

- Pentachloronitrobenzene in Organochlorine pesticide estimation in drinking water
- 2-nitrotoluene in Organophosphorous pesticide estimation in water.
- Phenylacetylene is used as IS is used as IS in BTX estimation using GC-MS
- n- propanol and t- butanal as IS in estimation of alcohol in blood using GC- MS.
- p-terphenyl is used as IS in PAH

Surrogate Standard:

It is chemically inert compound not expected to occur in an environmental sample. It should be added to each sample. It should be added to each sample, blank and matrix spike sample just prior to extraction or processing. The recovery of the surrogate standards is used to monitor for unusual matrix effects, grass sample processing error etc. Surrogate recovery is evaluated for acceptance by determining whether the measured concentration falls within acceptance limits.
Recommended surrogates for different analyte groups follows.

- 4,4'dichloro diphenyl for organochlorine pesticide in water
- 1,3- dimethyl 2 –nitrobenzene for organophosphorous pesticide in water
- Decafluorobiphenyl is used as surrogate standard in PAH estimation

Recommendation for storage of analytical reference standards-

- Select a solvent, such as isooctane or toluene that will dissolve the standard material and evaporate slowly.
- Store the standard is relatively large volume solution (50-100ml) to reduce the percentage volume loss to acceptable levels.
- Monitor the solvent loss by placing a mark on the side of the solution container and then discard the solution when 10% solvent has evaporated.
- Select a container, such as a screw –cap prescription bottle or a large volume volumetric flask that will not allow rapid solvent evaporation.
- Store the standard, away from light in refrigerator when not in use to reduce evaporation and reference material decomposition.
- Replace the easily decomposed reference standard solution at the recommended intervals.
- Check periodically solutions by comparison against fresh dilution of the stock solution for validity of reference standard.
- Don't store any standard solution of stock or intermediate for larger than one year.
- Working standard should be replaced six monthly.

Serial dilution of pesticide standard mixture-

All equipment used for tests must be calibrated before being put into service it chromatography and spectrometry method calibration can be done by use of CRM'S.

For chromatography system GC, GC-MS,HPLC etc. following procedure is adopted.

- Put the equipment system to get stabilize at the working condition (prechecked).
- See base line to be straight.
- Calculate the following for the performance of the column

Relative retention time (RRT)=Retention time of compound

Retention time of reference standard

LINEARITY GRAPH-After getting all the above parameter up to satisfaction the linearity graph with at least 5 concentrations should be drawn.

The above procedure must be done at interval of six months or whenever there any change in column or repair of the equipment is done.

Repeatability: Inject the standard three to five times and observe the variation in area of peaks. It should not be more than 3%.

Intermediate Check: Recheck the concentration of prepared working standard with freshly prepared working standards from stock solution to maintain confidence in calibration status.

Day to day calibration: In multiple residue analysis of pesticide or PAH it is not possible to draw calibration graph for each compound. It is done by injecting every day standard in the morning after getting the instrument completely stabilized. However the same standard may also be injected in evening if any unstablization observed in instruments. Sample peak height should not be much different as compare to standard. To minimize this either

Dilute the concentrate sample in the range of standard.

OR

Inject another set of standard solution, which is comparable to those of sample.

How to get confidence in the data or result obtained

- By recovery experiment
- Participating in intra-Laboratory Quality Assurance Programme
- Participation in Inter- Laboratory Quality Assurance Programme
- Participation in Proficiency Testing (PT) Programme

Proficiency Testing (PT) Programme:

Proficiency Testing (PT) in the use of inter laboratory test comparison to determine the performance of individual laboratories for specific tests / calibration.

Purposes:

- Check the consistency and comparability of data generated by the laboratories
- Support to accreditation bodies and regulatory authorities
- Demonstration of participating laboratories competence for customers satisfaction
- Evaluation of new methods against the existing standard methods.
- Participation in PT programmes provides the laboratories with an objective means of assessing and demonstrating the reliability of data which they are producing .

Recovery Experiment:

The analysts are always involved in the estimation of different analytes in different matrix using different techniques and equipment but it is very essential to know that either reported values are correct or not. It can be achieved by doing recovery experiment. Recovery experiments are done by spiking a known quantity in particular matrix before doing any process. It is to be done in replicate (minimum 7 along with three control).The spiking level should be minimum at LOQ level and at MRL(Maximum Residue Level) or permissible level.It is advisable to do at list three more level ie one below the LOQ ,second one 2 times of LOQ and third just above the MRL. The recovery of analytes depends upon the following:

- Nature of Matrix
- Spike concentration
- Techniques used
- Equipment used
- Total experience of particular chemist.
-

%Active / impurity content	Acceptable mean recovery (%)
> 10	98 - 102
> 1	90 - 110
0.1 - 1	80 - 120
< 0.1(1000ppm)	75 - 125

$$\% \text{ Recovery} = \frac{\text{Amount Recovered X 100}}{\text{Amount spiked}}$$

HOW TO CALCULATE THE SPIKING AMOUNT:

First of all note down the following information

a) Spiking Concentration required in ppm (ug/go rug/ml)

b) Weight(volume) of matrix in gram or ml

c) Number of replicate for spiking =7

d) Number of replicate for control(without any spiking)=3

Amount of analyte to be added= a X b = S ug

Now a standard of ppm concentration is taken which contain analyte in µg/ml may be take for spiking.

Volume(in ml) of spiking standard spiked in above matrix	=	Amount of analyte to be added µg / Concentration of standard in µg/ml

Example:

Spiking concentration required = 20 ppb = 0.02 ppm ie 0.02 µg/gm

Weight of sample (Meat)= 10g

Amount of analyte to be added= 0.02µg/g X 10g=0.2 µg

Standard concentration used for spiking=1ppm=1µg/ml

Volume of standard to be taken for spiking in 10 g sample= 0.2µg/1µg/ml =0.2ml

List Of suppliers of Standard / Certified Reference Materials

M/S Prochem India Pvt Ltd. P.O. Box 8061, No. 142, III floor 5th cross, Rajmahal Vilas Extension Banglore-560080, India Tel : 080-23364774, 23461256,23461257 Email : <**in@promochem.com**	Supelco M/S Sigma- Aldrich,31/1 Sitharamapalaya, Mahadevapur P.O. Banglore-560048 Tel: 080-51127272 FAX : 080-51127473 Email : India@sial.com
M/S Merck Ltd. 8th Floor, Godrej One, Pirojshanagar, Eastern Express Highway, Vikhroli (E) Mumbai - 400079	Chromatopak Analytical Instrumentation (India) Pvt. Ltd. Panchratna Apartment, 29-B Jawahar Nagar, Goregaon (West), Mumbai-400082 Email: chromopakb@vsnl.com

Measurement of Uncertainty in Chemical Analysis

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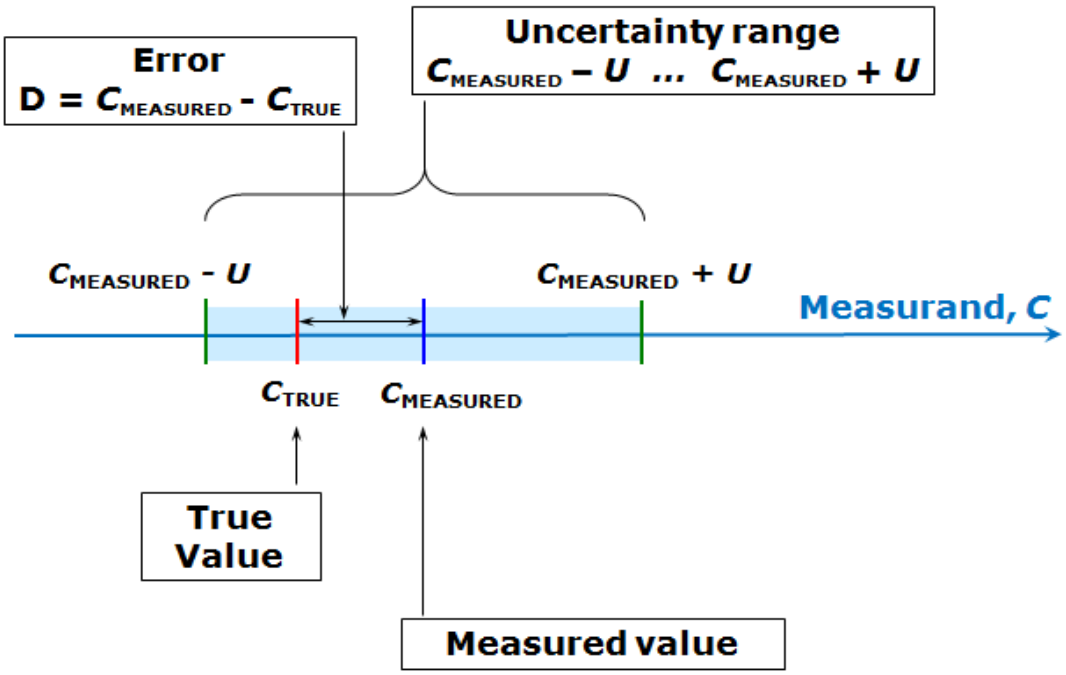
Measurement is a process of experimentally obtaining the value of a quantity. The quantity that we intend to measure is called **measure and**. In chemistry the measurand is usually the content (concentration) of some chemical entity (molecule, element, ion, etc) in some object. The chemical entity that is intended be determined is called **analyte**. Measurands in chemistry can be, for example, lead concentration in a water sample, content of pesticide thiabendazole in an orange or fat content in a bottle of milk. In the preceding example lead (element), ascorbic acid (molecule) and fat (group of different molecules) are the analytes. Water, orange and milk are **analysis objects** (or **samples** taken from analysis objects).

In principle, the aim of a measurement is to obtain the **true value** of the measurand. Every effort is made to optimize the **measurement procedure** (in chemistry chemical analysis procedure or analytical procedure in such a way that the measured value is as close as possible to the true value. However, our measurement result will be just an estimate of the true value and the actual true value will (almost) always remain unknown to us. Therefore, we cannot know exactly how near our measured value is to the true value – our estimate always has some uncertainty associated with it.

The difference between the measured value and the true value is called **error.** Error can have either positive or negative sign. Error can be regarded as being composed of two parts – **random error** and **systematic error** – which will be dealt with in more detail in coming lectures. Like the true value, also the error is not known to us. Therefore it cannot be used in practice for characterizing the quality of our measurement result – its agreement with the true value.

The quality of the measurement result, its **accuracy**, is characterized by **measurement uncertainty** (or simply **uncertainty**), which defines an interval around the measured value $C_{MEASURED}$, where the true valueC_{TRUE} lies with some probability. The

measurement uncertainty *U* itself is the half-width of that interval and is always non-negative. **[2]** The following scheme illustrates this:



The International Standard ISO/IEC 17025:2005 on "General Requirements for the Competence of Testing and Calibration Laboratories" has included a series of clauses on the estimation of measurement uncertainty for calibration and testing laboratories. It requests the assessment of uncertainty of test results during method validation and requires testing laboratories to have and apply procedures for estimating uncertainties of measurement in all test methods except when the test methods preclude such rigorous estimations. The word "uncertainty" means doubt, and thus in its broadest sense "uncertainty of measurement" means doubt about the validity of the result of a measurement. Measurement uncertainty is defined as "parameter, associated with the result of a measurement that characterizes the dispersion of the values that could reasonably be attributed to the measurand".

This definition is also consistent with other concepts of uncertainty of measurement, such as: - a measure of the possible error in the estimated value of the measurand as provided by the result of a measurement; - an estimate characterizing the range of values within which the true value of a measurand lies.

When uncertainty is evaluated and reported in a specified way, it indicates the level of confidence that the value actually lies within the range defined by the uncertainty interval.

Reasons for Estimating Uncertainty

- There is a growing awareness that analytical data for use in any decision process must be technically sound and defensible. Limits of uncertainty are required which need to be supported by suitable documentary evidence in the form of statistical control as for some kind of ‘quality assurance’. When a measurement process is demonstrated by such statistical control, the accuracy of the process can be implied to characterize the accuracy of all data produced by it.
- It is a recognized fact that any chemical analysis is subject to imperfections. Such imperfection gives rise to an error in the final test result. Some of these are due to random effects, typically due to unpredictable variations of influence quantities, such as fluctuations in temperature, humidity or variability in the performance of the analyst. Other imperfections are due to the practical limits to which correction can be made for systematic effects, such as offset of a measuring instrument, drift in its characteristics between calibrations, personal bias in reading an analogue scale or the uncertainty of the value of a reference standard.
- Every time a measurement is taken under essentially the same conditions. Random effects give rise to random errors from various sources and this affects the measured value. Repeated measurements will show variation and a scatter of test results on both sides of the average value. Statisticians say that random errors affect the precision, or reproducibility. A number of sources may contribute to this variability, and their influence may be changing continually. They cannot be completely eliminated but can be reduced by increasing the number of replicated analysis.
- Systematic errors emanate from systematic effects. They cause all the results to be in error in the same sense, i.e. either producing consistently higher or lower results than the true value. They remain unchanged when a test is repeated under the same conditions. These effects also cannot be eliminated but may be reduced or corrected with a correction factor if a systematic effect is recognized. In fact, systematic errors must be first dealt with before estimating any uncertainty in a chemical analysis.
- Hence, measurement uncertainty is a quantitative indication of the quality of the test result produced. It reflects how well the result represents the value of the quantity being measured. It allows the data users to assess the reliability of the result and have confidence in the comparability of results generated elsewhere on

the same sample or same population of the samples. Such confidence is important in the attempt to remove barriers to trade internationally.

- An understanding of the measurement uncertainty helps also in the validation of a new test method or a modified test method. One can suggest additional experiments to fine tune the test method if the uncertainty of the results is found to be large. One can also optimize the critical steps in a chemical analytical procedure in order to reduce uncertainty.
- By quoting measurement uncertainty, the laboratory operator reflects well on the technical competence of his laboratory staff performing the analysis and helps to communicate the limitations of test results to his customer.

Sources of Uncertainty in Chemical Measurement

There are many possible sources of uncertainty of measurement in testing, including but not limiting to:

a) Non-representative sampling - the sample analyzed may not be representative of the defined population, particularly when the it is not homogeneous in nature;
b) Non-homogeneity nature of the sample, leading to uncertainty in testing a sub-sample from the sample;
c) Incomplete definition of the measurand (e.g. failing to specify the exact form of the analyte being determined, such as Cr3+ and Cr6+);
d) Imperfect realization of the definition of the test method. Even when the test conditions are defined clearly, it may not be possible to produce these conditions in a laboratory; e) Incomplete extraction and pre-concentration of the test solution before analysis;
e) Contamination during sample and sample preparation;
f) Inadequate knowledge of the effects of environmental conditions on the measurement or imperfect measurement of environmental conditions;
g) Matrix effects and interference;
h) Personal bias in reading measurements (e.g. colour readings);
i) Uncertainty of weights and volumetric equipment
j) Uncertainty in the values assigned to measurement standards and reference
k) materials;
l) Instrument resolution, or discrimination threshold, or errors in the graduation
m) of the scale;

n) Approximations and assumptions incorporated in the measurement method and procedure;
o) Values of constants and other parameters obtained from external sources and used in the data reduction algorithm;
p) Random variation in repeated observations of the measurand under apparently identical conditions. Such random effects may be caused by short term environmental fluctuations (e.g. temperature, humidity, etc.) or variability between analysts.

It is to be noted these sources are not necessarily independent and, in addition, unrecognized systematic effects may exist that are not taken into accounts but which contributed to an error. However, such errors may be reduced, for example, from examination of the results of an inter-laboratory proficiency programme.

Evaluation Methods

The ISO Guide 98, ISO/TS 21748:2004 and the EURACHEM document have all adopted the approach of grouping uncertainty components into two categories based on their method of evaluation, i.e. *Type A* and *Type B* evaluation methods. 5.2 This categorization, based on the method of evaluation rather than on the components themselves, applies to uncertainty and is not substitutes for the words "random" and "systematic". It avoids certain ambiguities - a random component of uncertainty in one measurement may become a systematic component in another measurement that has, as its input, the result of the first measurement. For example, the overall uncertainty quoted on a certificate of calibration of an instrument will include the component due to random effects, but, when this overall value is subsequently used as the contribution in the evaluation of the uncertainty in a test using that instrument, the contribution would be regarded as systematic.

Type A evaluation of uncertainty is based on any valid statistical method in analysis of a series of repeated observations. The statistical estimated standard uncertainty is called, for convenience, a *Type A standard uncertainty*.

Component of *Type A* evaluation of standard uncertainty arises from random effect. The Gaussian or Normal Law of Error forms the basis of the analytical study of random effects. (See Appendix B)

It is a fact that the mean of a sample of measurement provides us with an estimate of the true value, μ of the quantity we are trying to measure. Since, however, the individual

measurements are distributed about the true value with a spread which depends on the precision; it is most unlikely that the mean of the sample is exactly equal to the true value of the population.

For this reason, it is more useful to give a range of values within which we are almost certain the true value lies. The width of the range depends on two factors. The first is the precision of the individual measurements, which in turn depends on the variance of the population. The second is the number of replicates made in the sample. The very fact that we repeat measurements implies that we have more confidence in the mean of several values than in a single one. Most people will feel that the more measurements we make, the more reliable our estimate of *μ,* the true value*.*

Type B evaluation is by means other than used for *Type A* such as:

- From data provided in calibration certificates and other reports;
- From previous measurement data;
- From experience with, or general knowledge of the behaviour of the instruments;
- from manufacturers' specifications;
- from all other relevant information.

Components evaluated using *Type B* methods are also characterized by estimated standard uncertainty.

When we are considering Type B uncertainty, we have to convert the quoted uncertainty to a standard uncertainty expressed as standard deviation. We can convert a quoted uncertainty that is a stated multiple of an estimate standard deviation to a standard uncertainty by dividing the quoted uncertainty by the multiplier.

Example:

A calibration report for reference weights states that the measurement uncertainty of a 1-gm weight is 0.1 mg at 2 standard deviations. The standard uncertainty is therefore 0.1 mg divided by 2 which gives 0.05 mg.

The quoted uncertainty can also be converted to a standard uncertainty from the knowledge of the probability distribution of the uncertainty. These probability distributions can be in the standard form of rectangular, triangular, trapezoidal and normal or Gaussian. Divide the quoted uncertainty by a factor which depends on the probability distribution.

The components, evaluated by either *Type A* or *Type B* methods, are combined to produce an overall value of uncertainty known as the combined standard uncertainty. An *expanded uncertainty* is usually required to meet the needs of industrial, commercial, health and safety, and other applications. It is obtained by multiplying the combined standard uncertainty by a *coverage factor, k*. The *k* value can be 2 for a 95% confidence level and 3 for a 99.7% confidence level. The expanded uncertainty defines an interval about the result of a measurement that may be expected to encompass a large fraction of the distribution of values that could reasonably be attributed to the measurand.

Structure of Analytical Procedure

Before the discussion on the methods for estimating uncertainty, it is helpful to first of all break down the analytical method into a set of generic steps in order to identify the possible sources of uncertainty:

a) Sampling
b) Sample preparation
c) Use of certified reference materials to the measuring system
d) Calibration of instrument
e) Analysis for data acquisition
f) Data processing
g) Presentation of results
h) Interpretation of results

Each of these steps can be further broken down by contributions to the uncertainty for each.

Reporting Uncertainty

The information necessary to document a measurement ultimately depends on its intended use but it should contain enough information to allow the result to be reevaluated if new information or data become available. A complete analysis report should include the followings:

a) a description of the methods used to calculate the result and its uncertainty;
b) the values and sources of all corrections and constants used in both the
c) calculation and the uncertainty analysis;
d) a list of all the components of uncertainty with full documentation on how each was evaluated.

The data and analysis should be presented in such a manner that its important steps can be readily followed and if necessary repeated.

Reporting Expanded Uncertainty

Unless it is required otherwise, the result should be reported together with the expanded uncertainty, *U,* calculated using a coverage factor k = 2. The calculated expanded measurement uncertainty, *U*, represents half of the measurement uncertainty interval. The following standardised format is usually applied to express the entire measurement uncertainty interval, accompanied with a statement:

(Analyte): Result x (units) ± *U* (units)

_ *The reported measurement uncertainty is an expanded measurement uncertainty according to this Guide, calculated using 2 as the coverage factor [which gives a confidence level of approximately 95%].*

Note that the texts within square brackets [ ] may be omitted or abbreviated in a suitable manner.

*Example:Total Oil Content : 9.80 ± 0.15% w/w**

* *The reported uncertainty is an expanded uncertainty calculated using a coverage factor of 2 which gives a confidence level of approximately 95%.*

Modern Analytical techniques for the samples preparation of organic pollutants (SPME/SPE/DLLME)

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Online demonstration through Video will organised

Demo: Processing/extraction for analysis of organic pollutants (SPE/SPME/DLLME)

Scope: Analysis of Organic Pollutants in different environmental and biological matrices by using solid phase extraction (SPE), solid phase microextraction (SPME) and dispersive liquid-liquid microextraction

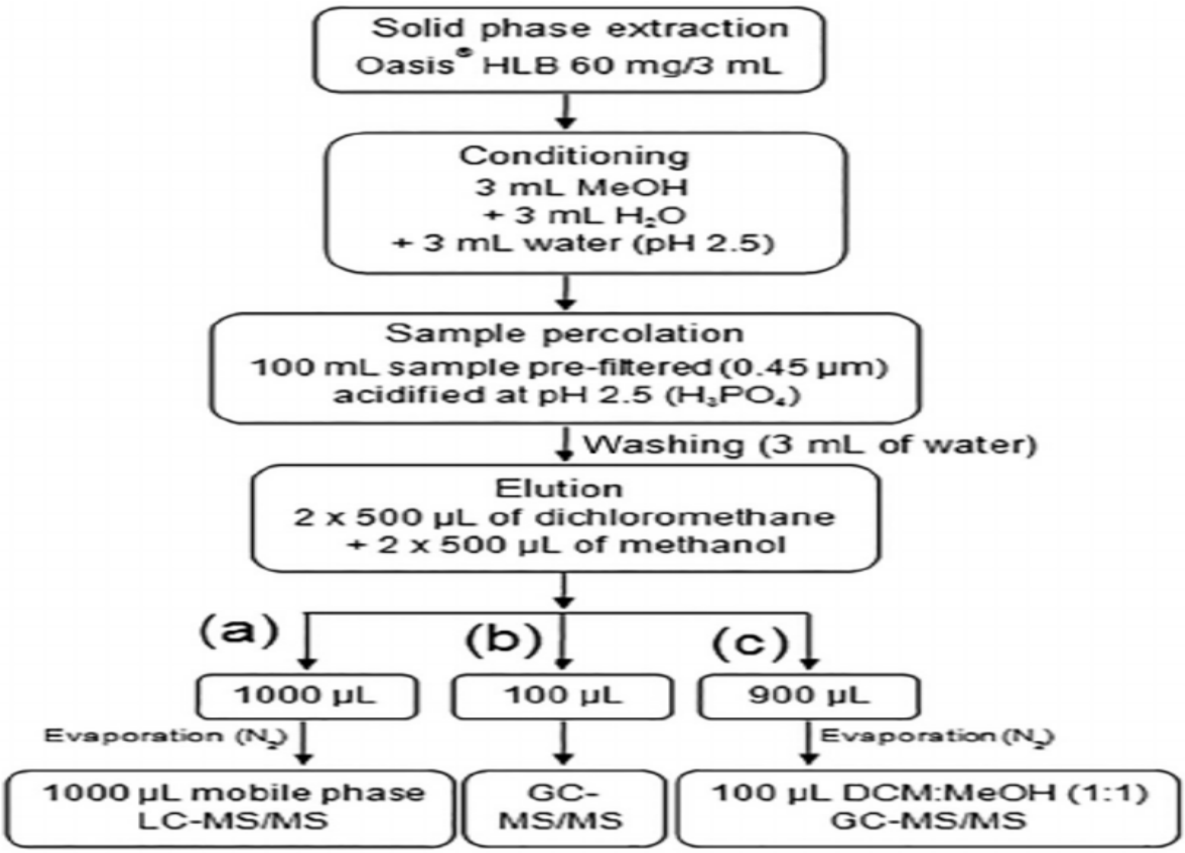
Requirements:

1. Solid Phase Extraction: SPE Setup, SPE Cartridge, Glass tubes, DCM, Methanol
2. Solid Phase Microextraction: SPME Fibers, SPME Setup,
3. Dispersive Liquid-Liquid Micro Extraction: Extraction solvent, Dispersive solvent, Vortex Machine, Centrifuge

Procedure:

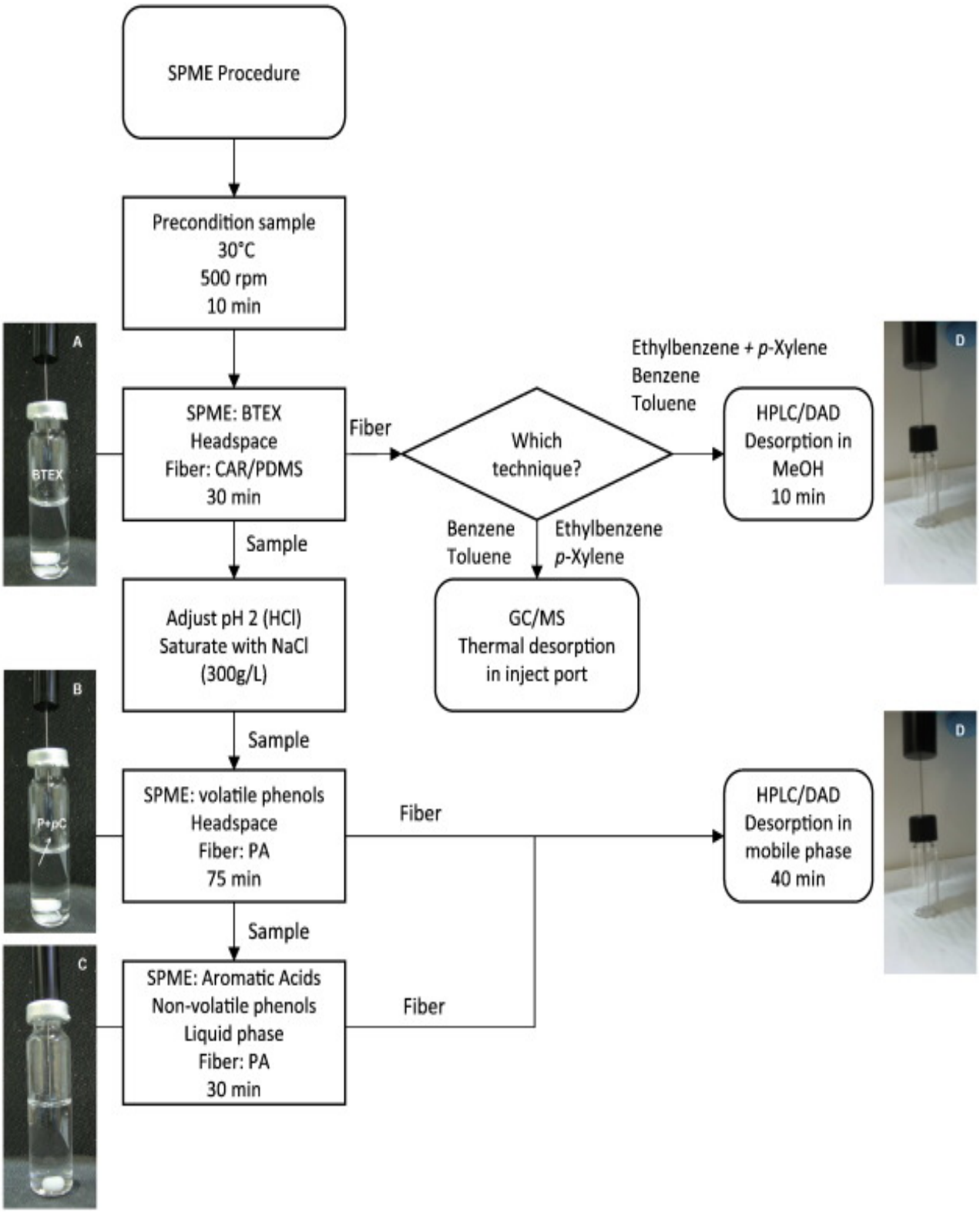
1. **Solid Phase Extraction (SPE):**
   i. Take 1 Lt of water sample, filter the sample through 0.22µ Whatman filter paper
   ii. Make sure that SPE set up should be arranged in proper way
   iii. Fit Empty syringe to SPE vacuum manifold
   iv. Wash all the tubings with Water and solvent
   v. Keep SPE cartridges to SPE setup
   vi. Equilibrate the cartridges with water and solvent
   vii. Pass the water samples through SPE cartridges at optimized flow
   viii. Ensure that there is no water molecule present in cartridges
   ix. Then keep the cartridges at -20°C for 4-5 Hrs
   x. Elute the targeted analytes in appropriate extraction solvent
   xi. Evaporate the extractantupto dryness and make up the volume with diluent

xii. Inject 1µL of extractant in to chromatographic system



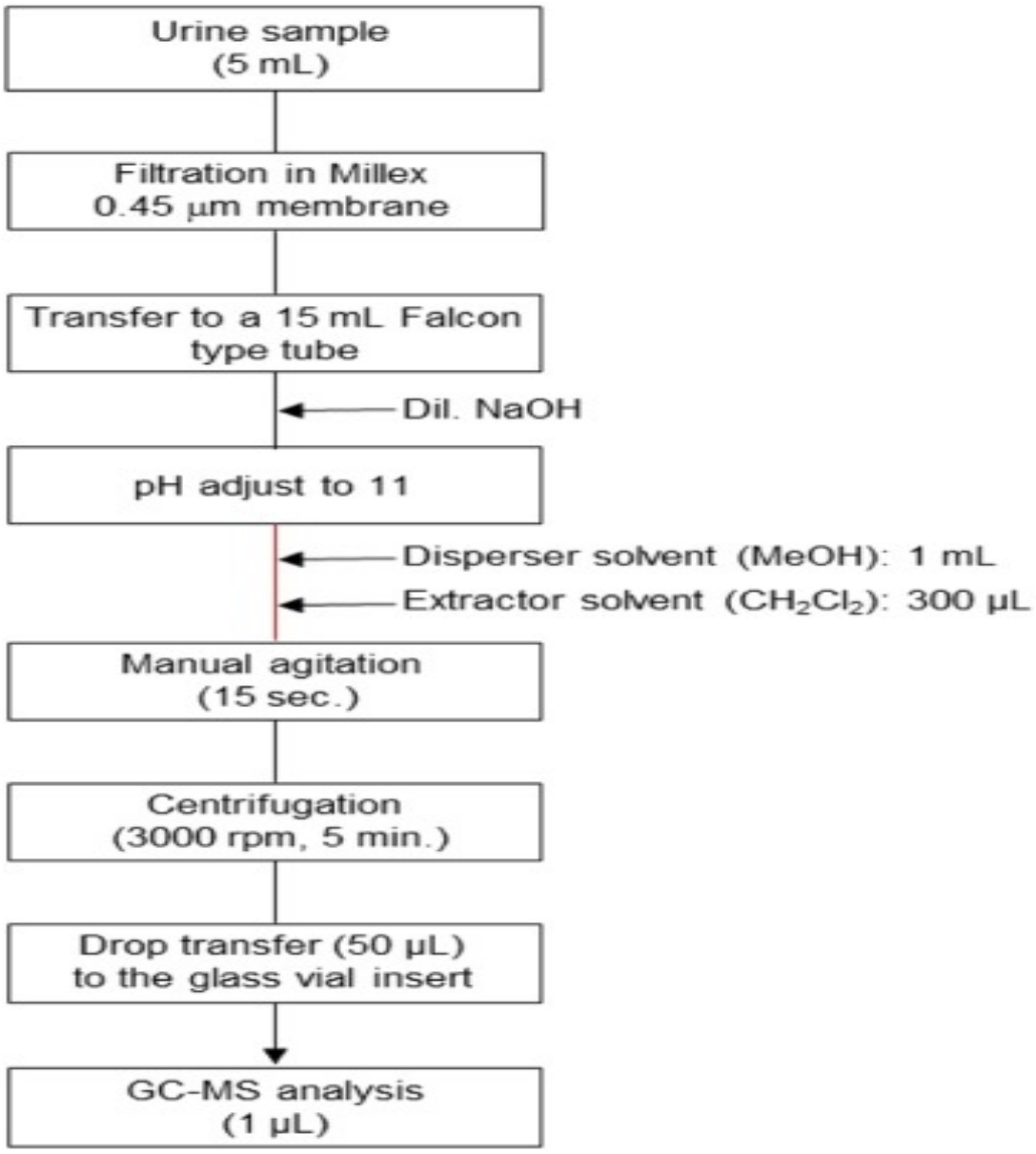
2. **Solid Phase Micro Extraction (SPME):**

i. Incubate the sample at pre-defined temperature, rotations and time
ii. Precondition the Headspace fiber (DVB/CAR/PDMS) at optimized temperature at particular time interval
iii. Adjust the pH of the sample by using HCl
iv. Keep the sample on Hot Plate and fix the SPME fiber
v. Extract the targeted analytes by using appropriate fiber at pre-defined time
vi. Remove the SPME set up from the sample
vii. Insert the SPME set up in GC injector by thermal desorption at pre-defined temperature
viii. Run the sample on chromatographic system


SPME Procedure
Precondition sample
30°C
500 rpm
10 min
A
BTEX
SPME: BTEX
Headspace
Fiber: CAR/PDMS
30 min
Fiber
Which technique?
Ethylbenzene + p-Xylene
Benzene
Toluene
HPLC/DAD
Desorption in
MeOH
10 min
D
Sample
Benzene
Toluene
Ethylbenzene
p-Xylene
GC/MS
Thermal desorption
in inject port
Adjust pH 2 (HCl)
Saturate with NaCl
(300g/L)
B
P+pC
Sample
SPME: volatile phenols
Headspace
Fiber: PA
75 min
Fiber
HPLC/DAD
Desorption in
mobile phase
40 min
D
Sample
C
SPME: Aromatic Acids
Non-volatile phenols
Liquid phase
Fiber: PA
30 min
Fiber

3. Dispersive Liquid-Liquid Microextraction (DLME)

i. Before going to conduct the experiment, optimize type of extraction solvent and dispersion solvent
ii. Take 1-2 mL of sample in 5 mL centrifuge tube
iii. Inject pre-mix solution (defined volume) of extraction and dispersion solvent forcefully into sample
iv. Vortex the sample of defined (optimized) rpm and time
v. Centrifuge the sample at constant rpm of predefined time
vi. Remove the extractant by using syringe
vii. Add diluent to make the sample non-viscous
viii. Inject 1µL of sample in to chromatographic system



Demo: Calibration of Laboratory Glassware's

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The volume of volumetric analysis rests, as in gravimetric analysis, fundamentally on the accuracy of the instruments employed to determine the volume of a standard solution required to react with a known quantity of an unknown substance. This applies also to the measurement of an aliquot of the solution of the sample to be analyzed. Where it is directed that the quantity be "accurately measured" the apparatus must be chosen and used with care. A burette should be of such size that the titrant volume represents not less than 30% of the nominal volume. Where less than 10 ml of titrant is to be measured a 10 ml micro burette is generally required.

Instrument Used

The instruments used are volumetric flasks, burettes, transfer pipettes and measuring pipettes. They are made of glass and available in various sizes.

Requirements: Analytical balance, weights box, 1mL, 5ml, 10mL, 50mL and 100mL volumetric flask, and 1mL, 5mL, 10mL graduated pipettes

Procedure:

1. Switch on the power of the balance
2. Kept the balance for 15-20 min for stabilization
3. The reading should be 0.0000 and no fluctuation should be there
4. Temperature of the balance room should be in between 25+2°C
5. Kept the small beaker on the PAN and the reading of the beaker should be tarred i.e. 0.0000
6. Fill the glassware (volumetric flask/pipettes/ measuring cylinder) with Milli Q water and pour the water in beaker and take the reading
7. This process should be done at least four times
8. Before taking the next reading, every time the previous water should be cleaned with tissue paper from the glassware

9. Then put the reading in the excel sheet on the computer
10. Take the mean of the values
11. Take the standard deviation
12. Then calculate the corrected value by using following formula

Corrected volume = Correction factor + Mean value
Difference = Corrected volume-certificate volume

Calibration

Volumetric glassware is normally calibrated at 27°C. However, the temperature generally specified for measurement of volume in analytical operation of the pharmacopoeia, unless otherwise stated, is 25 *°C.* This discrepancy is in-consequent as long as the room temperature in the laboratory is reasonably constant and is around 27*°C.* There are two grades of apparatus available designated as –

Class A is intended for use in work of highest accuracy.
Class B apparatus may be employed in routine work.

Volumetric Apparatus

The use of volumetric glass ware requires the setting or reading of a meniscus. The reading or setting is made on the lowest point of the meniscus, except for those liquids that are so strongly coloured that they are opaque. Source of error is the determination of the exact bottom of the meniscus and the second source of error is parallax.
Burettes and measuring pipettes should be checked at a minimum of five points. For a **50 ml** burette the intervals **0-10 ml**, **0-20 ml**, **0-30 ml** and **0-40 ml** and **0-50 ml** should be checked.

For this, fill the burette to just above the zero line with distilled water at room temperature and determine the temperature (T) of the water. Set the meniscus at exactly zero, and wipe off the excess water from the tip with a clean piece of filter paper. Place a tared stoppered flask under the burette and deliver the interval to be tested into tared stoppered flask and reweigh the flask. The true volume of interval is calculated from the apparent weight of the water at temperature T using Table 1 & 2.

Apparent weight of water of fixed volume = X

From Table 1 or 2, Correction factor at particular temp. x volume of water/100 =X'

$$\text{Volume of apparatus} = \frac{X + X'}{1.000028} = \text{ml}$$

Volumetric flask up to 100 ml capacity can be calibrated in the same manner. Weigh the volumetric flask on analytical balance. Fill up with double distilled water up to the mark. Again weigh the flask. True volume of flask is calculated. Similarly as described in case of pipette and burette.

An alternate following formula may be used using Table 1 and 2 given in IS: 8897-1978

Volume of apparatus (ml) = Wt. of water of fixed volume of apparatus(g) + correction factor of temp. for particular volume at water temperature (Table 1) + Correction factor of pressure at laboratory atmospheric pressure (if required Table 2)

Note: In our laboratory the change in atmospheric pressure is not going to influence the calibration. Table 2 of IS:8897-1978 suggests that the change in atmospheric pressure of +10 mm Hg may change the volume maximally only in third decimal place.

The result obtained should be in comparison with Table 3.

Example of glassware calibration:

24.04.2019		**Glassware calibration**		**Temp -26.4 ℃**		**Correction factor= 0.415/100ml**					**Atm Press= 755.31 mm Hg**		
Item	**Volume**	**Certificate**		**wt of Dw**	**wt of Dw**	**Wt of Dw**	**Wt of Dw**	**Wt of DW**	**Mean**	**SD**	**CF as per**	**Corrected**	**Difference**
		code	**Value**								**volume**	**volume**	
Volumetric Flasks	**100 ml**	3230	99.991	99.5678	100.0044	100.0021	99.9986	99.9829	99.9111	0.19213	0.415	100.3262	0.3352
	100 ml	3288	100.048	100.0022	99.9768	99.9956	99.6874	99.6753	99.8674	0.17020	0.415	100.2825	0.2345
	50 ml	379	49.981	49.8625	49.8989	49.8674	49.8543	49.9892	49.8944	0.05559	0.2075	50.1020	0.1210
	50 ml	373	50.002	50.0024	49.9928	49.9878	49.8569	49.8752	49.9430	0.07076	0.2075	50.1505	0.1485
	10 ml	192	9.998	9.9922	9.9345	9.9689	9.9925	9.9089	9.9594	0.03685	0.0415	10.0009	0.0029
	10 ml	129	9.995	9.9923	9.9878	9.9884	9.9756	9.9158	9.9719	0.03202	0.0415	10.0135	0.0185

24.04.2019		Glassware calibration		Temp -26.4 ℃		Correction factor= 0.441/100ml					Atm Press= 755.31 mm Hg		
Item	Vol.	Certificate		wt of Dw	wt of Dw	Wt of Dw	Wt of Dw	Wt of DW	Mean	SD	CF as per	Corrected	Difference
		Code	Value								volume	volume	
Pipettes	10 ml	155	10.017	9.9945	9.9826	9.9923	9.9812	9.9885	9.9878	0.0058	0.0441	10.0319	0.0149
	10 ml	148	9.966	9.9758	9.962	9.9682	9.9725	9.9896	9.9736	0.0103	0.0441	10.0177	0.0517
	5 ml	3142	4.9901	4.9926	4.9869	4.9925	4.9872	4.9856	4.9889	0.0033	0.0220	5.0110	0.0209
	5 ml	3171	4.994	4.9902	4.9951	4.9865	4.9789	4.9815	4.9864	0.0065	0.0220	5.0084	0.0145
	2 ml	1501	1.99	1.9985	1.9912	1.9903	1.9952	1.989	1.9928	0.0039	0.0088	2.0016	0.0117
	2 ml	1506	1.9919	1.9859	1.9824	1.9829	1.9832	1.9903	1.9849	0.0032	0.0088	1.9937	0.0019
	1 ml	1530	0.9974	0.9925	1.0068	0.9899	0.9903	0.9905	0.994	0.0072	0.0044	0.9984	0.0010
	1 ml	1545	1.0007	0.9856	1.0009	1.0016	0.9789	1.003	0.994	0.0110	0.0044	0.9984	-0.0023

Table 1:Corrections for determining the true capacities of glass vessels from the weight of water in air (Soft glass, coefficient of cubical expansion 0.000010/^{o}C) Indicated capacity 100 ml.

Temperature °C	Tenths of degrees									
	0	1	2	3	4	5	6	7	8	9
15	0.207	0.208	0.21	0.211	0.212	0.213	0.215	0.216	0.217	0.219
16	0.22	0.221	0.223	0.224	0.225	0.227	0.228	0.23	0.231	0.232
17	0.234	0.235	0.237	0.238	0.24	0.241	0.243	0.244	0.246	0.247
18	0.249	0.25	0.252	0.253	0.255	0.257	0.258	0.26	0.261	0.263
19	0.265	0.266	0.268	0.27	0.272	0.273	0.275	0.277	0.278	0.28
20	0.282	0.284	0.285	0.287	0.289	0.291	0.293	0.294	0.296	0.298
21	0.3	0.302	0.304	0.306	0.308	0.31	0.312	0.314	0.315	0.317
22	0.319	0.321	0.323	0.325	0.327	0.329	0.331	0.333	0.336	0.338
23	0.34	0.342	0.344	0.346	0.348	0.35	0.352	0.354	0.357	0.359
24	0.361	0.363	0.365	0.368	0.37	0.372	0.374	0.376	0.379	0.381
25	0.383	0.386	0.388	0.39	0.392	0.395	0.397	0.399	0.402	0.404
26	0.406	0.409	0.411	0.414	0.416	0.418	0.421	0.423	0.426	0.428
27	0.431	0.433	0.436	0.438	0.44	0.443	0.446	0.448	0.451	0.453
28	0.456	0.458	0.461	0.463	0.466	0.469	0.471	0.474	0.476	0.479
29	0.482	0.484	0.487	0.49	0.492	0.495	0.498	0.501	0.503	0.506
30	0.509	0.511	0.514	0.517	0.52	0.522	0.525	0.528	0.531	0.534
31	0.536	0.539	0.542	0.545	0.548	0.551	0.554	0.556	0.559	0.562
32	0.565	0.568	0.571	0.574	0.58	0.58	0.583	0.586	0.589	0.592

Table 2: Corrections for determining the true capacities of glass vessels from the weight of water in air (Borosilicate glass, coefficient of cubical expansion 0/000010/^{o}C) Indicated capacity 100 ml.

Temperature °C	Tenths of degrees									
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
15	0.2	0.201	0.202	0.204	0.205	0.207	0.208	0.21	0.211	0.212
16	0.214	0.215	0.217	0.218	0.22	0.222	0.223	0.225	0.226	0.228
17	0.229	0.231	0.232	0.234	0.236	0.237	0.239	0.241	0.242	0.244
18	0.246	0.247	0.249	0.251	0.253	0.254	0.256	0.258	0.26	0.261
19	0.263	0.265	0.267	0.269	0.271	0.272	0.274	0.276	0.278	0.28
20	0.282	0.284	0.286	0.288	0.29	0.292	0.294	0.296	0.298	0.3
21	0.302	0.304	0.306	0.308	0.31	0.312	0.314	0.316	0.318	0.32
22	0.322	0.324	0.327	0.329	0.331	0.333	0.335	0.338	0.34	0.342
23	0.344	0.346	0.349	0.351	0.353	0.355	0.358	0.36	0.362	0.365
24	0.367	0.369	0.372	0.374	0.376	0.379	0.381	0.383	0.386	0.388
25	0.391	0.393	0.396	0.398	0.4	0.403	0.405	0.408	0.41	0.413
26	0.415	0.418	0.42	0.423	0.426	0.428	0.431	0.433	0.436	0.438
27	0.441	0.444	0.446	0.449	0.452	0.454	0.457	0.46	0.462	0.465
28	0.468	0.47	0.473	0.476	0.479	0.481	0.484	0.487	0.49	0.492
29	0.495	0.498	0.501	0.504	0.506	0.509	0.512	0.515	0.518	0.521
30	0.524	0.526	0.529	0.532	0.535	0.538	0.541	0.544	0.547	0.55
31	0.553	0.556	0.559	0.562	0.565	0.568	0.571	0.574	0.577	0.58
32	0.583	0.586	0.589	0.592	0.595	0.598	0.602	0.605	0.608	0.611

Table 3: Capacity tolerance for volumetric flasks, pipettes and burettes as per IS specification

Volumetric flasks: IS 915:1975

Nominal capacity, **ml**	**5**	**10**	**25**	**50**	**100**	**250**	**500**	**1000**
Tolerance, + ml								
Class A	0.02	0.02	0.03	0.04	0.06	0.1	0.15	0.2
Class B	0.04	0.04	0.06	0.08	0.15	0.2	0.3	0.8

One-Mark Pipettes: IS 1117: 1975

Nominal capacity, **ml**	**1**	**2**	**5**	**10**	**20**	**25**	**50**	**100**
Tolerance + ml								
Class A	0.01	0.01	0.02	0.02	0.03	0.03	0.04	0.06
Class B	0.02	0.02	0.03	0.04	0.05	0.06	0.08	0.12

Graduated Pipettes: IS 4162:1967

Nominal capacity, ml	**1**	**2**	**5**	**10**	**25**
Sub divisional, + ml	0.01	0.02	0.05	0.10	0.2
Class A 0.006	0.01	0.03	0.05	0.1	
Class B 0.02	0.06	0.10			

Burettes: IS 1997: 1967

Nominal capacity, ml	**10**	**25**	**50**	**100**
Sub divisional, + ml	0.05	0.05	0.1	0.1
Class A 0.01	0.03	0.05	0.1	
Class B 0.02	0.06	0.1	0.2	

Reference:

Standard Methods of Chemical Analysis, Sixth Edition, Volume II, Part A., F.J. Welcher, 1975 and Indian Pharmacopoeia, 1996. Indian Standard IS: 8897-1978 Table for calibration and method of verification of volumetric glassware.

Demo:Calibration of Analytical Balance

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Introduction:

In all analytical procedures weighing is the most crucial step and one the most common sources of error. These errors can be reduced if proper precautions are taken during the operation of analytical balance. This standard operating procedure describes procedures for use, routine maintenance and calibration of **"AND GR-200"** electronic analytical balance.

Instrument used:

Analytical balance, weights box, 200g, 100g, 50g, 20g, 10g, 5g, 2g, 1g, 0.5g, 0.2g, 0.1g, 0.05g, 0.02g, 0.01g, 0.005g, 0.002g, 0.001g weights.

Use

This balance is suitable for weighing in the range of 5 mg to 180 g with the reported sensitivity 0.1 mg.

Pre-operational checking

Before making the weighing check the following:

i) Check the balance is located in a place of even temperature with no direct sunlight.
ii) It should be placed away from any vibration.
iii) Balance must be perfectly leveled by centering the spirit level bubble using the two level screws at the bottom of the balance.
iv) Ensure that all the doors of the balance are properly closed. The glass wares and chemicals taken out from refrigerator or oven should be allowed to reach at room temperature for half an hour or depending upon the requirement.

v) A camel hair brush of appropriate size should be used for cleaning the balance pan and its surrounding area inside the balance chamber to ensure that these are free from dust and chemicals.

Calibration

Periodical calibration of the balance is required. Sometimes due to power fluctuation or due to any other cause balance displays an error message, under such conditions the balance must be calibrated again as per the instruction manual of the balance

The balance is also calibrated with standard weights calibrated by NPL, New Delhi starting from 10 mg to 100 gm periodically. The uncertainty of the weight taken is at the fourth place of decimal.

Operational Procedure:

i) Connect the balance with a proper voltage (220-240ºC) power switch and switch it ON. Allow the balance to warm up for 10 minutes.
ii) Check that the weighing pan is clean.
iii) Press the ON/OFF switch, and check the digits on screen which should be 0.0000. If not, Press tare to get 0.0000.
iv) Do not put the object directly in the pan. Solids are always weighed in weighing bottles, watch glass, aluminum foil or beaker or any other container and liquids are also weighed in a suitable container. Use of filter paper is strictly prohibited for weighing chemicals.
v) Check that the container used for weighing is free from moisture.
vi) All objects to be weighed must be at ambient temperature. Objects can not be weighed accurately above room temperature as they create current of air that interferes with the readings of balance.
vii) If any drift occurs in weighing, check the following and eliminate the errors.
    a) Balance door are open.
    b) Temperature variation within the weighing environment
    c) Vibration from laboratory operation.
    d) Balance not properly leveled
    e) Sample and balance temperature are not same.
    f) Hydroscopic chemicals

Calibration Procedure:

- Take the standard weight, of 100 grams put it on the pan and note the reading on the digital display of the balance. Put the same weight at 5 different points on the pan and note the reading.
- Repeat the process with other standard weights of 50 grams, 20 grams, 10 grams and 5 grams. .
- Repeat this process under identical manner with standard weights in the lower range i.e. milligram range, taking weights of 500 mg, 200 mg, 100 mg, 50 mg,
- 20 mg and 10 mg. Take five readings of each weight keeping the weight at different points on the pan. .
- Note the deviation and calculate the uncertainty taking into account the uncertainty of standard weights as mentioned in the calibration certificate
- The value of three limits the standard deviation of the reproducibility of the balance divided by the amount of weight should be less than 0.001. (Ref: Indian Pharmacopeia, 2007 21.
- Using the balance *for* routine weighing, the same procedure should be adopted.

Procedure for calibration of Electronic Balance using a set of standard weights (NPL Calibrated):

- Switch on the Balance
- Give it a warm-up time of about 10 minutes
- Check the level of the balance with the help of built-in spirit level.
- Level it with the help of leveling screws provided.
- Calibrate the balance with built in calibration of the balance as per the instruction manual of the balance. .
- Before starting the calibration with the standard weights, proper care should be taken to clean the pan with a soft brush.
- The standard weights should be cleaned before use with lint free cloth.
- Always use a clean forceps while putting and removing the standard weights from the weight box or pan of the balance.

Data Recording

The recording of data should be done by the individual on their study register. In any case it should not be recorded on the loose sheets.

Safety Precaution

Laboratory coats should be worn at all times while handling the balance. Face masses, gloves and goggles should be worn while handling toxic chemicals.

TEST DATA WORK SHEET

S.No.	VALUE OF STANDARD WEIGHT	Readings at different points of PAN					Mean ± SD	3 x SD/weight (should be < 0.001)
		w-1	w-2	w-3	w-4	w-5		
1.	100 Grams							
2.	50 Grams							
3.	20 Grams							
4.	10 Grams							
5.	5 Grams							
6.	500 mgs.							
7.	200 mgs.							
8.	100 mgs.							
9.	50 mgs.							
10.	20 mgs.							
11.	10 mgs.							
12.	5 mgs.							

Example: Analytical Balance AND-GR 200

	200 G	100 G	50 G	20 G	10 G	5 G	2 G	1 G
	200.0031	100.0017	50.0011	20.001	10.0002	5.0001	2	1.0001
	200.0014	100.0009	50.0008	20.0009	10.0001	5	2	1.0001
	200.0025	100.0014	50.001	20.0009	10.0001	5.0002	2	1.0001
	200.0047	100.0024	50.0015	20.0011	10.0002	5.0002	2	1.0001
	200.0034	100.0017	50.0012	20.001	10.0002	5.0002	2	1.0002
MEAN	200.0030	100.0016	50.00112	20.00098	10.00016	5.00014	2	1.00012
SD	0.0012	0.00054	0.00026	0.00008	0.00005	0.00009	0.00000	0.00004
Performance	0.0000	0.00002	0.00002	0.00001	0.00002	0.00005	0.00000	0.00013

	0.5 G	0.2 G	0.1 G	0.05 G	0.02 G	0.01 G	0.005 G	0.002 G	0.001 G
	0.5	0.2	0.1000	0.0501	0.0200	0.0100	0.0050	0.0020	0.0009
	0.5	0.2	0.1000	0.0501	0.0200	0.0100	0.0050	0.0020	0.0009
	0.5	0.199	0.1000	0.0501	0.0200	0.0100	0.0050	0.0020	0.0009
	0.5001	0.2	0.1001	0.0501	0.0200	0.0100	0.0050	0.0020	0.0009
	0.5	0.2	0.1000	0.0501	0.0200	0.0100	0.0050	0.0020	0.0009
MEAN	**0.50002**	**0.1998**	**0.1000**	**0.0501**	**0.0200**	**0.0100**	**0.0050**	**0.0020**	**0.0009**
SD	0.00004	0.00045	0.00004	0.00000	0.00000	0.0000	0.0000	0.0000	0.0000
Performance	0.00027	0.00671	0.00134	0.00000	0.00000	0.0000	0.0000	0.0000	0.0000

Gas Chromatography Technique and its Applications in Environmental Samples Analysis

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A **gas chromatography** is a chemical analysis instrument for separating chemicalsin a complex sample. A gas chromatograph uses a flow-through narrow tube known as the *column*, through which different chemical constituents of a sample pass in a gas stream (carrier gas, *mobile phase*) at different rates depending on their various chemical and physical properties and their interaction with a specific column filling, called the *stationary phase*. As the chemicals exit the end of the column, they are detected and identified electronically. The function of the stationary phase in the column is to separate different components, causing each one to exit the column at a different time (*retention time*). Other parameters that can be used to alter the order or time of retention are the carrier gas flow rate, and the temperature.

Principle of Gas chromatography

In a GC analysis, a known volume of gaseous or liquid analyte is injected into the "entrance" (head) of the column, usually using a microsyringe (or, solid phase microextractionfibers, or a gas source switching system).

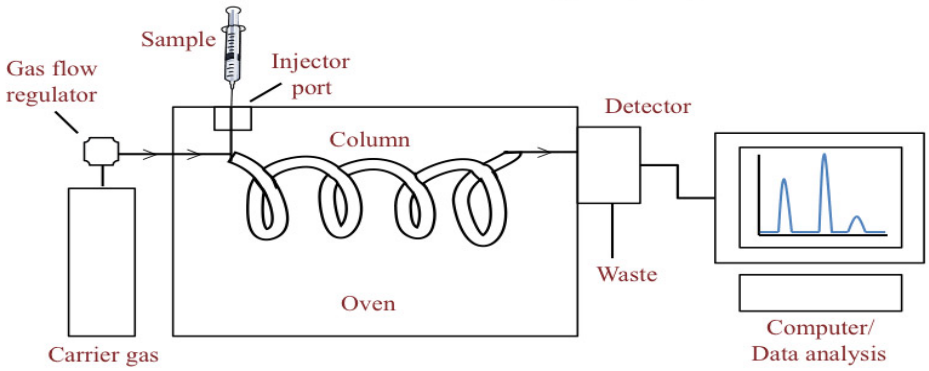
The equilibrium for gas chromatography is partitioning, and the components of the sample will partition (i.e. distribute) between the two phases: the stationary phase and the mobile phase.

Compounds that have a greater affinity for the stationary phase spend more time in the column and thus elute later and have a longer **retention time (Rt)** than samples that have a higher affinity for the mobile phase.

Affinity for the stationary phase is driven mainly by intermolecular interactions and the polarity of the stationary phase can be chosen to maximize interactions and thus the separation.Ideal peaks are Gaussian distributions and symmetrical, because of the random nature of the analyte interactions with the column.

- The separation is due to partitioning the sample between the gas and a thin layer of a nonvolatile liquid held on a solid support.
- A sample containing the solutes is injected into a heated block (injector) where it is immediately vaporized and flows through the carrier gas stream into the column inlet.
- The solutes are adsorbed by the stationary phase and then desorbed by a fresh carrier gas.
- The process is repeated in each plate as the sample is moved toward the outlet.

- Each solute will travel at its own rate through the column.
- Their bands will separate into distinct zones depending on the partition coefficients, and band spreading.
- The solutes are eluted one after another in the increasing order of their kd, and enter into a detector attached to the exit end of the column.
- Here they register a series of signals resulting from concentration changes and rates of elution on the recorder as a plot of time versus the composition of carrier gas stream.
- The appearance time, height, width, and area of these peaks can be measured to yield quantitative data.



Components of gas chromatography

Physical components

1. Autosamplers

The autosampler provides the means to introduce automatically a sample into the inlets. Manual insertion of the sample is possible but is no longer common. Automatic insertion provides better reproducibility and time-optimization. Different kinds of autosamplers exist. Autosamplers can be classified in relation to sample capacity (auto-injectors VS autosamplers, where auto-injectors can work a small number of samples), to robotic technologies (XYZ robot VS rotating/SCARA-robot – the most common), or to analysis

- Liquid
- Static head-space by syringe technology
- Dynamic head-space by transfer-line technology
- SPME

Inlets

The **column inlet** (or *injector*) provides the means to introduce a sample into a continuous flow of carrier gas. The inlet is a piece of hardware attached to the *column head*.

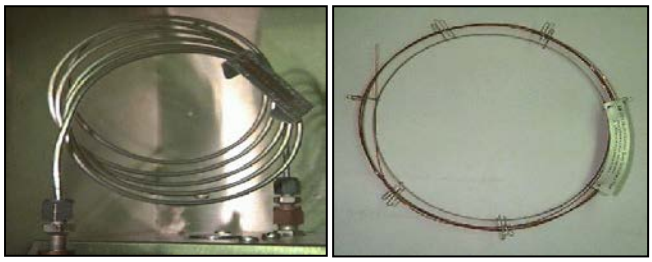
Common inlet types are:

- **S/SL (Split/Splitless) injector**; a sample is introduced into a heated small chamber via a syringe through a *septum* - the heat facilitates volatilization of the sample and sample matrix. The carrier gas then either sweeps the entirety (*splitless* mode) or a portion (*split* mode) of the sample into the column. In split mode, a part of the sample/carrier gas mixture in the injection chamber is exhausted through the *split vent*.
- **On-column inlet**; the sample is here introduced in its entirety without heat.
- **PTV injector**; Temperature-programmed sample introduction was first described by Vogt in 1979. Originally Vogt developed the technique as a method for the introduction of large sample volumes (up to 250 µL) in capillary GC. Vogt introduced the sample into the liner at a controlled injection rate. The temperature of the liner was chosen slightly below the boiling point of the solvent. The low-boiling solvent was continuously evaporated and vented through the split line. Based on this technique, Poy developed the Programmed Temperature Vaporising injector; PTV. By introducing the sample at a low initial liner temperature many of the disadvantages of the classic hot injection techniques could be circumvented.
- **Gas source inlet** or **gas switching valve**; gaseous samples in collection bottles are connected to what is most commonly a six-port *switching valve*. The carrier gas flow is not interrupted while a sample can be expanded into a previously evacuated *sample loop*. Upon switching, the contents of the sample loop are inserted into the carrier gas stream.
- **P/T (Purge-and-Trap) system**; An inert gas is bubbled through an aqueous sample causing insoluble volatile chemicals to be purged from the matrix. The volatiles are 'trapped' on an absorbent column (known as a trap or concentrator) at ambient temperature. The trap is then heated and the volatiles are directed into the carrier gas stream. Samples requiring preconcentration or purification can be introduced via such a system, usually hooked up to the S/SL port.
- SPME (solid phase microextraction) offers a convenient, low-cost alternative to P/T systems with the versatility of a syringe and simple use of the S/SL port.

2. Columns

Two types of columns are used in GC:

Packed columns are 1.5 - 10 m in length and have an internal diameter of 2 - 4 mm. The tubing is usually made of stainless steel or glass and contains a *packing* of finely divided, inert, solid support material (eg. diatomaceous earth) that is coated with a liquid or solid stationary phase. The nature of the coating material determines what type of materials will be most strongly adsorbed. Thus numerous columns are available that are designed to separate specific types of compounds.



Packed column **Capillary column**

Capillary columns have a very small internal diameter, on the order of a few tenths of millimeters, and lengths between 25-60 meters are common. The inner column walls are coated with the active materials (WCOT columns), some columns are quasi solid filled with many parallel micropores (PLOT columns). Most capillary columns are made of fused-silica with a polyimide outer coating. These columns are flexible, so a very long column can be wound into a small coil.

New developments are sought where stationary phase incompatibilities lead to geometric solutions of parallel columns within one column. Among these new developments are:
Internally heated *microFAST* columns, where two columns, an internal heating wire and a temperature sensor are combined within a common column sheath (microFAST).

Micropacked columns (1/16" OD) are column-in-column packed columns where the outer column space has a packing different from the inner column space, thus providing the separation behaviour of two columns in one. They can be easily fit to inlets and detectors of a capillary column instrument.

Column temperature and temperature program

The column(s) in a GC are contained in an oven, the temperature of which is precisely controlled electronically. (When discussing the "temperature of the column," an analyst is technically referring to the temperature of the column oven. The distinction, however, is not important and will not subsequently be made in this article.)

The rate at which a sample passes through the column is directly proportional to the temperature of the column. The higher the column temperature, the faster the sample moves through the column. However, the faster a sample moves through the column, the less it interacts with the stationary phase, and the less the analytes are separated.

In general, the column temperature is selected to compromise between the length of the analysis and the level of separation.

A method which holds the column at the same temperature for the entire analysis is called "isothermal." Most methods, however, increase the column temperature during the analysis, the initial temperature, rate of temperature increase (the temperature "ramp") and final temperature is called the "**temperature program**."

A temperature program allows analytes that elute early in the analysis to separate adequately, while shortening the time it takes for late-eluting analytes to pass through the column.

The **temperature-dependence** of molecular adsorption and of the rate of progression along the column necessitates a careful control of the column temperature to within a few tenths of a degree for precise work. Reducing the temperature produces the greatest level of separation, but can result in very long elution times. For some cases temperature is ramped either continuously or in steps to provide the desired separation. This is referred to as a **temperature program**. Electronic pressure control can also be used to modify flow rate during the analysis, aiding in faster run times while keeping acceptable levels of separation.

The **choice of carrier gas** (*mobile phase*) is important, with hydrogen being the most efficient and providing the best separation. However, helium has a larger range of flowrates that are comparable to hydrogen in efficiency, with the added advantage that helium is non-flammable, and works with a greater number of detectors. Therefore, helium is the most common carrier gas used.

3. Detectors

A number of detectors are used in gas chromatography. The most common are

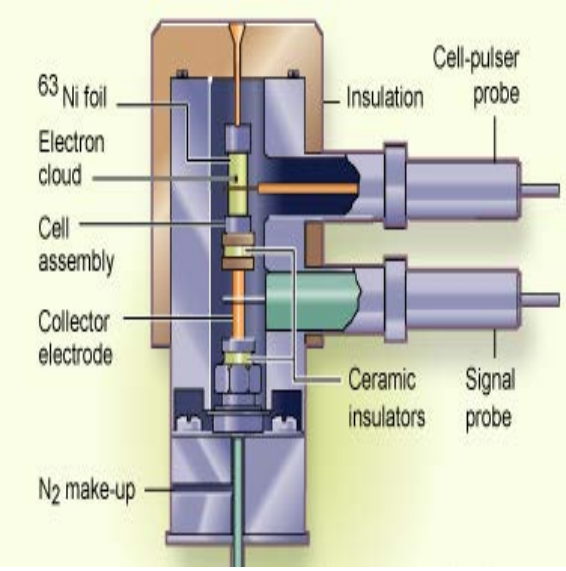
- *Electron Capture Detector (ECD)*
- *Flame Ionization Detector (FID)*
- *Nitrogen-Phosphorous Detector (NPD)*

Electron capture detector (ECD)

The **electron capture detector** (ECD) was invented in 1957, by Dr. James E. Lovelock. It is a device for use in gas chromatography that can detect tiny amounts of chemical compounds in the atmosphere and elsewhere. The electron capture detector is used in detecting electron-absorbing components in the output stream of a gas chromatograph. The ECD uses a radioactive Beta particle (electrons) emitter -- a typical source contains a metal foil holding 10 millicuries of Nickel-63. The electrons formed are attracted to a positively charged anode, generating a steady current. As the sample is carried into the detector by a stream of nitrogen or a 5% methane, 95% argon mixture, analyte molecules capture the electrons and reduce the current between the collector anode and a cathode. The analyte concentration is thus proportional to the degree of electron capture, and this detector is particularly sensitive to halogens, organometallic compounds, nitriles, or nitro compounds.

Sensitivity

The ECD is 10-1000 times more sensitive than an FID, and one million times more sensitive than a TCD, but has a limited dynamic range and finds its greatest application in analysis of halogenated compounds. The detection limit for electron capture detectors is 5 femtograms per second (fg/s), and the detector commonly exhibits a 10,000-fold linear range. This made it possible to detect halogenated compounds such as pesticides and CFCs, even at levels of only one part per trillion (ppt), thus revolutionizing our understanding of the atmosphere and pollutants.

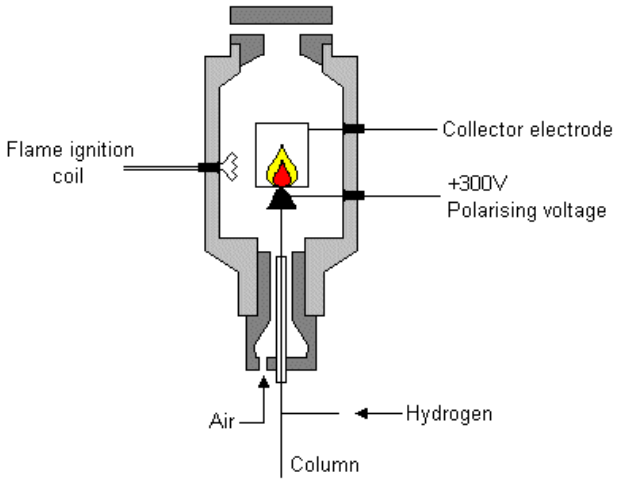


FLAME IONIZATION DETECTOR (FID):

The flame ionization detector (FID) is the most sensitive gas chromatographic detector for hydrocarbons such as butane or hexane. With a linear range for 6 or 7 orders of magnitude (10^6 to 10^7) and limits of detection in the low picogram or femtogram range, the FID is the most widely and successfully used gas chromatographic detector for volatile hydrocarbons and many carbon containing compounds.

This detector is very sensitive towards organic molecules (10-12 g/s = 1 pg/s, linear range: 10^6-10^7), but relative insensitive for a few small molecules i.e., N_2, NO_x, H_2S, CO, CO_2, H_2O. If proper amounts of hydrogen/air are mixed, the combustion does not afford any or very few ions resulting in a low background signal. If other carbon containing components,are introduced to this stream, cations will be produced in the effluent stream. The more carbon atoms are in the molecule, the more fragments are formed and the more sensitive the detector is for this compound. Unfortunately, there is no direct relationship between the number of carbon atoms and the size of the signal. As a result, the individual response factors for each compound have to be experimentally determined for each instrument. Due to the fact that the sample is burnt (pyrolysis), this technique is not suitable for preparative GC. In addition, several gases are usually required to operate a FID: hydrogen, oxygen (or compressed air), and a carrier gas.

The Flame Ionisation Detector



The effluent from the column is mixed with hydrogen and air, and ignited. Organic compounds burning in the flame produce ions and electrons which can conduct electricity through the flame. A large electrical potential is applied at the burner tip, and a collector electrode is located above the flame. The current resulting from the pyrolysis of any organic compounds is measured. FIDs are mass sensitive rather than concentration sensitive; this gives the advantage that changes in mobile phase flow rate do not affect the detector's response. The FID is a useful general detector for the analysis of organic compounds; it has high sensitivity, a large linear response range, and low noise. It is also robust and easy to use, but unfortunately, it destroys the sample.

Usually the small diameter capillary is fitted directly into the bottom of the detector's flame jet. The gaseous eluents from the column are mixed with separately plumbed in hydrogen and air and all are burned on the jet's tip. After the fuel (H2) and oxidant (O_2 in air) are begun, the flame is lit using an electronic ignitor, actually an electrically heated filament that is turned on only to light the flame.

The charged particles created in that combustion process create a current between the detector's electrodes. One electrode is actually the metallic jet itself, another is close by and above the jet. The gaseous products leave the detector chamber via the exhaust. The detector housing is heated so that gases produced by the combustion (mainly water) do not condense in the detector before leaving the detector chimney.

Mechanism: Compounds are burned in a hydrogen-air flame. Carbon containing compounds produce ions that are attracted to the collector. The number of ions hitting the collector is measured and a signal is generated.

- Mass sensitive detector
- Response depends on conducting power of ions or electrons produced on burning of organic compounds in the flame

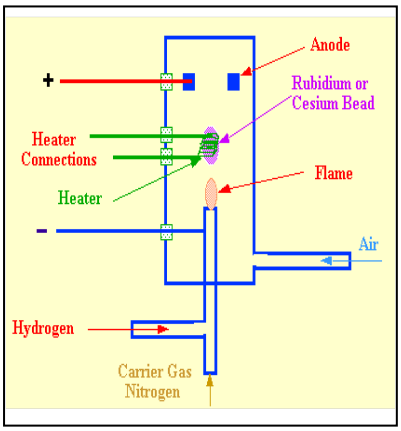
- Selective detector but sample detected must be combustible
- Large linear dynamic range (107)
- No response to inorganic and permanent gases such as CO, CO2, NH3, CS2, N_2, etc.
- It is the most widely used detector in Gas Chromatography

Selectivity: Compounds with C-H bonds. A poor response for some non-hydrogen containing organics (e.g., hexachlorobenzene). Sensitivity is 0.1-10 ng

Nitrogen Phosphorus Detector (NPD)

The nitrogen phosphorus detector (NPD) (sometimes called the *thermionic detector*) is a very sensitive, specific detector the design of which, is based on the FID. Physically the sensor appears to be very similar to the FID but, in fact, operates on an entirely different principle.

NPD sensor differs from that of the FID by a rubidium or cesium chloride bead contained inside a heater coil situated close to the hydrogen jet. The bead is situated above a jet and heated by a coil, over which the nitrogen carrier gas mixed with hydrogen passes. If the detector is to respond to both nitrogen and phosphorus, then the *hydrogen* flow should be *minimal* so that the gas does not ignite at the jet. If the detector is to respond to phosphorus, only, however, a large flow of hydrogen can be used and the mixture burnt at the jet. The heated alkali bead emits electrons by thermionic emission which are collected at the anode and provides background current through the electrode system. When a solute that contains nitrogen or phosphorusiseluted, thepartiallycombustednitrogenandphosphorusmaterials are adsorbed on the surface of the bead.



The Nitrogen Phosphorus Detector

Carrier gas selection and flow rates

Typical carrier gases include helium, nitrogen, argon, hydrogen and air. Which gas to use is usually determined by the detector being used, for example, a DID requires

helium as the carrier gas. When analyzing gas samples, however, the carrier is sometimes selected based on the sample's matrix, for example, when analyzing a mixture in argon, an argon carrier is preferred, because the argon in the sample does not show up on the chromatogram. Safety and availability can also influence carrier selection, for example, hydrogen is flammable, and high-purity helium can be difficult to obtain in some areas of the world. (See: Helium--occurrence and production.)

The purity of the carrier gas is also frequently determined by the detector, though the level of sensitivity needed can also play a significant role. Typically, purities of 99.995% or higher are used. Trade names for typical purities include "Zero Grade," "Ultra-High Purity (UHP) Grade," "4.5 Grade" and "5.0 Grade."

The carrier gas flow rate affects the analysis in the same way that temperature does (see above). The higher the flows rate the faster the analysis, but the lower the separation between analytes. Selecting the flow rate is therefore the same compromise between the level of separation and length of analysis as selecting the column temperature.

Many modern GCs, however, electronically measure the flow rate, and electronically control the carrier gas pressure to set the flow rate. Consequently, carrier pressures and flow rates can be adjusted during the run, creating pressure/flow programs similar to temperature programs.Inlet types and flow rates

The choice of inlet type and injection technique depends on if the sample is in liquid, gas, adsorbed, or solid form, and on whether a solvent matrix is present that has to be vaporized. Dissolved samples can be introduced directly onto the column via a COC injector, if the conditions are well known; if a solvent matrix has to be vaporized and partially removed, a S/SL injector is used (most common injection technique); gaseous samples (e.g., air cylinders) are usually injected using a gas switching valve system; adsorbed samples (e.g., on adsorbent tubes) are introduced using either an external (on-line or off-line) desorption apparatus such as a purge-and-trap system, or are desorbed in the S/SL injector (SPME applications).

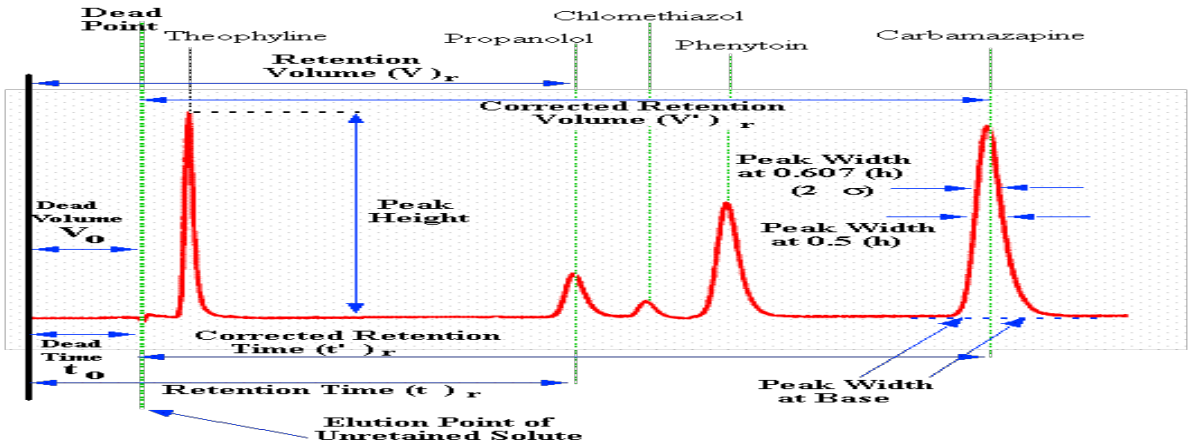
Sample size and injection technique

The rule of gas chromatography

The real chromatographic analysis starts with the introduction of the sample onto the column. The development of capillary gas chromatography resulted in many practical problems with the injection technique. The technique of on-column injection, often used with packed columns, is usually not possible with capillary columns. The injection system, in the capillary gas chromatograph, should fulfil the following two requirements:

1. The amount injected should not overload the column.
2. The width of the injected plug should be small compared to the spreading due to the chromatographic process. Failure to comply with this requirement will reduce the separation capability of the column. As a general rule, the volume injected,

V_{inj}, and the volume of the detecor cell, V_{det}, should be about 1/10 of the volume occupied by the portion of sample containing the molecules of interest (analytes) when they exit the column.



The Nomenclature of a Chromatogram.

Peak	The portion of a differential chromatogram recording the detector response while a single component is eluted from the column.
Baseline	is any part of the chromatogram where only mobile phase is emerging from the column.
Peak maximum	Highest point of the peak.
Injection point	point in time/position time when/where the sample is placed on the column.
Dead point	position of the peak-maximum of an unretained solute.
Dead time **(t_o)**	is the time elapsed between the *injection point* and the *dead point*.
Dead volume **(V_o)**	volume of mobile phase passed through the column between the *injection point* and the *dead point.* $V_o = Qt_o$ where Q is the flow rate in ml/min.
Retention time **(t_r)**	time elapsed between the *injection point* and the *peak maximum.* Each solute has a characteristic retention time.
Retention volume **(V_r)**	volume of mobile phase passed throughthe œolumnbetweenthe *injection point* and the *peak maximum.*$V_r = Qt_r$ where Q is the flow rate in ml/min.
Correctedretention volume **(v'_r)**	volume of mobile phase passed throughthe œolumnbetweenthe *dead point* and the *peak maximum.* It will also be the *retention volume* minus the *dead volume.* Thus, $V'_r = V_r - V_o = Q(t_r - t_o)$ where Q is the flow rate in ml/min.
Peak height **(h)**	Distance between the *peak maximum* and the *base line* geometricallyproduced beneath the peak.
Peak width **(w)**	Distance between each side of a peak measure at 0.6065 of the peak height (*ca* 0.607h). The peak width measured at this height is equivalent to two standard deviations (2s) of the Gaussian curve and thus has significance when dealing with chromatography theory.
Peak width at the base **(w_b)**	Distance between the intersections of the tangents drawn to the sides of the peak and the *peak base* geometrically produced. The peak width at the base is equivalent to four standard deviations (4s) of the Gaussian curve and thus also has significance when dealing with chromatography theory.

Applications

Qualitative analysis:

Generally chromatographic data is presented as a graph of detector response (y-axis) against retention time (x-axis). This provides a spectrum of peaks for a sample representing the analytes present in a sample eluting from the column at different times. Retention time can be used to identify analytes if the method conditions are constant. Also, the pattern of peaks will be constant for a sample under constant conditions and can identify complex mixtures of analytes. In most modern applications however the GC is connected to a mass spectrometer or similar detector that is capable of identifying the analytes represented by the peaks.

Quantitative analysis:

The area under a peak is proportional to the amount of analyte present. By calculating the area of the peak using the mathematical function of integration, the concentration of an analyte in the original sample can be determined. Concentration can be calculated using a calibration curve created by finding the response for a series of concentrations of analyte, or by determining the relative response factor of an analyte. The relative response factor is the expected ratio of an analyte to an internal standard (or external standard) and is calculated by finding the response of a known amount of analyte and a constant amount of internal standard (a chemical added to the sample at a constant concentration, with a distinct retention time to the analyte).In most modern GC-MS systems, computer software is used to draw and integrate peaks, and match MS spectra to library spectra.

Advantages

- The use of longer columns and higher velocity of carrier gas permits the fast separation in a matter of a few minutes.
- Higher working temperatures and the possibility of converting any material into a volatile component make gas chromatography one of the most versatile techniques.
- GC is popular for environmental monitoring and industrial applications because it is very reliable and can be run nearly continuously.
- GC is typically used in applications where small, volatile molecules are detected and with non-aqueous solutions or non-polar molecules.

Limitations

- Compound to be analyzed should be stable under GC operation conditions.
- They should have a vapor pressure significantly greater than zero also the compounds to be less than 1,000 Da, to get vaporize.
- The samples are also required to be salt-free; they should not contain ions.
- Very minute amounts of a substance can be measured, but it is often required that the sample must be measured in comparison to a sample containing the pure, suspected substance known as a reference standard.

DEMO: Preparation of samples for the estimation of organic pollutants (Pesticides, PAHs in water, air and soil) and analysis of samples on GCMS/MS

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Scope: Analysis and determination of organic pollutants in different environmental matrices by using gas chromatography tandem mass spectrometry

Requirements: Organic solvents (Dichloromethane, Ethyl acetate, n-hexane), separating funnel, separating funnel stand, activated charcoal tubes, acetonitrile, DCM, Methanol

Apparatus: Air sampling apparatus capable of running at a flow rate of 0.2-0.4 lpm, GC-FID, Ultra sonicator, centrifuge, GCMS/MS

Procedure:

Liquid-Liquid extraction for Water samples:

1. Keep the separating funnel on stand
2. Take 1 Lt of water sample in 2 Lt separating funnel
3. Add 25mg of NaCl and shaken for 2 mins
4. Add 50 mL of DCM/n-hexane
5. Shaken well separating funnel of defined time interval
6. Keep the separating funnel for stabilization
7. Separate the organic solvent in to 250 mL conical flask
8. Repeat the process for two times more
9. Mix all the portions of extractant in 250 mL conical flask
10. Add anhydrous sodium sulphate in to the conical flask
11. Transfer the extract into another 250 mL conical flask
12. Evaporate the sample upto dryness by using rotatory evaporator
13. Made up the volume up to 5 mL by using diluent in 5 mL volumetric flask
14. Inject 1 µL of sample on chromatographic system

Soxhlet extraction method for soil samples

1. Weigh 10g dried soil
2. Pack the sample in thimble (filter paper) is placed in main chamber of soxhlet
3. Add 150 mL of dichloromethane in the round bottom flask
4. Put the round bottom flask in the heating mantle and assembled the soxhlet with the condenser
5. Set the temperature of heating mantle to 30-40°C
6. Open the tap connected to the condenser for inlet and outlet
7. Run the soxhlet apparatus for 16 hours
8. After completing the extraction, take the extract from the RBF after rinsing the chamber by DCM twice
9. Pas the DCM extract sample through anhydrous sodium sulphate bed
10. Evaporate the sample upto dryness on chiller rotatory evaporator
11. Make the sample in 10 mL volumetric flask by DCM
12. If colour/turbidity is appear then the sample is clean by column using activated Florisil and anhydrous sodium sulphate through eluting solvent (n-hexane: DCM: Ethyl acetate) in the ration of 70:15:15
13. Evaporate the sample upto dryness by using rotatory evaporator
14. Make up the sample in 5 mL of volumetric flask with diluent
15. Inject 1 µL of sample on chromatographic system

Procedure for BTX extraction from ambient air

1. Air is drawn through the charcoal tube at 0.2-0.4 lpm for 30-60 mins
2. The contents of the tube are thereafter emptied into a 15 mL centrifuge tube and filled upto 5 cm with ACN
3. The tubes are ultrasonicated for 15 mins taking care to prevent sampling heating
4. The tubes are centrifuged at 10000 rpm for 15 mins at 4°C to ensure setting of charcoal
5. Depending on instrument feasibility i.e. whether ACN or Methanol is to be used as solvent in GC, 2 mL sample is either directly syringe filtered into GC Vials or solvent is switched to Methanol and syringe filtered & filled into vials for further analysis

Analysis of B(a)P in Ambient Air

a. Extraction: Filter papers (half of all the filters papers collected in a day) are cut into strips using scissors and transfer to 250 ml beaker. Add ~50 ml. of Toluene (GC/HPLC grade). These samples are extracted with toluene using ultra sonic bath for about 30 minutes. Repeat the procedure twice (50ml x 2 times) for complete extraction. Alternatively, sample can be extracted using soxhlet extraction apparatus for about 8 hr. with Toluene and repeat it twice.

b. Filtration: Filter the extracted samples with Whatman filter paper no.41 containing 2 gm of anhydrous Sodium Sulphate (to remove moisture).

c. Concentration: After filtration, the filtrate is concentrated using Rotary vacuum evaporator to 2ml final volume

d. Clean-up with silica Gel: To clean up the impurities, pass 2 ml of concentrated sample through silica gel column (pre conditioned, 60-80 mesh, and 200-250mm×10 mm with Teflon stopcock). After cleaning add 5ml cyclohexane and collect the elute in 25 ml beaker. Repeat the process for at least 3 times and collect it in the same beaker. Alternatively Solid Phase Extraction (SPE) may be used for clean up the impurities of sample.

e. Re-concentration with rotary vacuum evaporator: filter extraction with ultrasonicator the cleaned up extract/filtrate (approximately 17 ml) is further concentrated using rotary evaporator and it is evaporated to nearly dryness with nitrogen.
f. Final Sample volume: The dried sample is re-dissolved in 1ml of toluene and transfer into 4 or 5 ml amber vials final analysis.

g. Extracted Sample Storage: Cover/Cap the sample vials /tubes and mark with necessary identification. Keep it in refrigerator at 4°C prior to the analysis ready to Inject B(a)P Samples

h. Analysis: Instrument Set-Up GC Conditions: Injector: 300°C FID Temp: 320°C Column: Ultra -2 (25m Length, 320µm diameter, 0.17µ) or equivalent Oven: 120°C →2 min hold → 7°C/min → 300°C → 10 min hold Run Time: 37.71 minutes Carrier gas flow (N2): 0.50 ml/min Gases for FID Flame: H_2 flow: 40 ml/min Zero grade air flow: 400 ml/min

Preparation of samples for the estimation of organic pollutants (Pesticides, PAHs in water, air and soil) and analysis of samples on GCMS/MS

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Online demonstration through Video will organised

Principles of High Performance Liquid Chromatography

Dr. D. K. Patel, Senior Principal Scientist
Analytical Chemistry Section, Regulatory Toxicology Group
CSIR-Indian Institute of Toxicology

Chromatography is a dynamic physicochemical method of separation in which the components to be separated are distributed between two phases, one of which is stationary [the *stationary phase*] while the other [the *mobile phase*] moves relative to the stationary phase.

HPLC is liquid chromatography which has been optimized to provide rapid high resolution separations. It evolved over nearly a century from the early work of Tswett in the late 1900s to the highly sophisticated reliable and fast liquid chromatography (LC) techniques in common use today. High-performance liquid chromatography (HPLC) is an analytical technique to separate, identify, and quantify components in a mixture.

Types of HPLC

1. Normal-Phase HPLC

The column is filled with tiny silica particles, and a non-polar solvent, for example, hexane. Non-polar compounds in the mixture will pass more quickly through the column, as polar compounds will stick longer to the polar silica than non-polar compounds.
An elution procedure in which the stationary phase is more polar than the mobile phase is called Normal-phase HPLC.

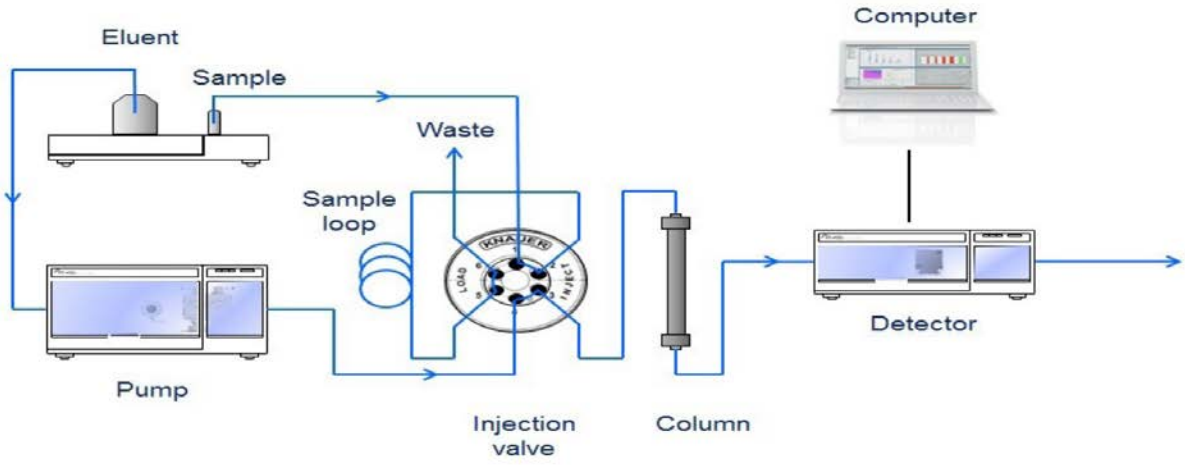
2.Reversed-Phase HPLC

The column is filled with silica particles which are modified to make them non-polar. This is done by attaching long hydrocarbon chains (8–18 C atoms) to its surface. A polar solvent is used, for example, a mixture of water and an alcohol such as methanol. Polar compounds in the mixture will pass more quickly through the column because a strong attraction occurs between the polar solvent and the polar molecules in the mixture.

An elution procedure used in liquid chromatography in which the mobile phase is significantly more polar than the stationary phase, *e.g.* a microporous silica-based material with alkyl chains chemically bonded to its accessible surface. This liquid chromatography is in contrast to Normal-phase HPLC is called *reversed-phase chromatography*.

Major HPLC components and their functions

1. Pump:
2. Injector:
3. Column:
4. Detector:
5. Computer:



1. HPLC Pumps

The role of the pump is to force a liquid (called the mobile phase) through the liquid chromatograph at a specific flow rate, expressed in milliliters per min (mL/min).

Because of the small particles used in modern HPLC, modern LC pumps need to operate reliably and precisely at pressures of 10,000 p.s.i. or at least 6,000 p.s.i. To operate at these pressures and remain sensibly inert to the wide variety of solvents used HPLC pumps usually have sapphire pistons, stainless steel cylinders and return valves fitted with sapphire balls and stainless steel seats. This level of constancy is required because most HPLC detectors are flow sensitive and errors in quantization will result from changes in flow rate.

- Normal flow rates in HPLC are in the 1- to 2-mL/min range.
- Typical pumps can reach pressures in the range of 6000-9000 psi (400- to 600-bar).
- During the chromatographic experiment, a pump can deliver a constant mobile phase composition (isocratic) or an increasing mobile phase composition (gradient).

Flow Rate

The volume of mobile phase passing through the column in unit time. In HPLC systems, the flow rate is set by the controller for the solvent delivery system [pump]. Flow rate accuracy can be checked by timed collection and measurement of the effluent at the column outlet. Since a solvent's density varies with temperature, any calibration or flow rate measurement must take this variable into account. Most accurate determinations are made, when possible, by weight, not volume.

Uniformity [precision] and *reproducibility* of flow rate is important to many LC techniques, especially in separations where *retention times* are key to analyte identification, or in *gel-permeation chromatography* where calibration and correlation of retention times are critical to accurate molecular-weight-distribution measurements of polymers.

Often, separation conditions are compared by means of *linear velocity*, not flow rate. The linear velocity is calculated by dividing the flow rate by the cross-sectional area of the column. While flow rate is expressed in volume/time [*e.g.,* mL/min], linear velocity is measured in length/time [*e.g.,* mm/sec].

Isocratic Elution

A procedure in which the composition of the mobile phase remains constant during the elution process.

Mobile Phase [Eluate, Eluent]

A fluid that percolates, in a definite direction, through the length of the stationary-phase sorbent bed. The mobile phase may be a liquid [*liquid chromatography*] or a gas [*gas chromatography*] or a supercritical fluid [*supercritical-fluid chromatography*]. In gas chromatography the expression *carrier gas* may be used for the mobile phase. In elution chromatography, the mobile phase may also be called the *eluent*, while the word *eluate*is defined as the portion of the mobile phase that has passed through the sorbent bed and contains the compounds of interest in solution.

HPLC Gradient Mixers

HPLC gradient mixers must provide a very precise control of solvent composition to maintain a reproducible gradient profile. This can be complicated in HPLC by the small elution volumes required by many systems. It is much more difficult to produce a constant gradient when mixing small volumes then when mixing large volumes. For low pressure systems this requires great precision in the operation of the miniature mixing valves used and low dispersion flows throughout the mixer. For multi-pump high pressure systems it requires a very precise control of the flow rate while making very small changes of the flow rate.

2. HPLC Sample Valves

Sample valves come between the pump and the column it follows that HPLC sample valves must also tolerate pressures upto 10000 p.s.i. For analytical HPLC, the sample volume should be selectable from sub- micro litre to a few micro litres, whereas in preparative HPLC the sample volume may be even greater than 10 ml. To maintain system efficiency the sample valve must be designed to have very low dispersion characteristics, this is true not only for flow dispersion but also for the less obvious problems of dispersion caused by sample adsorption/desorption on valve surfaces and diffusion of sample into and out of the mating surfaces between valve moving parts. It goes without saying that the valves must deliver a very constant sample size but this is usually attained by the use of a constant size sample loop.

- The injector serves to introduce the liquid sample into the flow stream of the mobile phase.
- Typical sample volumes are 5 to 20microliters (µL).
- The injector must also be able to withstand the high pressures of the liquid system.
- An autosampler is the automatic version for when the user has many samples to analyze or when manual injection is not practical.

3. HPLC Columns

It is one of the two phases forming a chromatographic system. It may be a solid, a gel, or a liquid. If a liquid, it may be distributed on a solid. This solid may or may not contribute to the separation process. The liquid may also be chemically bonded to the solid [*bonded phase*] or immobilized onto it [*immobilized phase*]. This is the Stationary Phase.

HPLC columns are packed with very fine particles (usually a few microns in diameter). The very fine particles are required to attain the low dispersion that give the high plate counts expected of modern HPLC. Plate counts in excess of 25,000 plates per column are possible with modern columns, however, these very high efficiencies are very rarely found with real samples because of the dispersion associated with injection valves, detectors, data acquisition systems and the dispersion due to the higher molecular weight of real samples as opposed to the common test samples.

Theoretical plates

$N=16(tr/wb)^2 = 5.54[tr/wh]$

A column efficiency value of 500 theoratical plates per foot for ppDDT is considered to be of minimal in terms of the generally expected peak resolution .it is 3,000 or more for 6 feet glass column. For 15 cm silica gel column show15000 theoretical plates

Peak resolution factor $R=1.18(Wrb – Wra)/Wha+ Whb$

It should be>1.0

Packing these small particles into the column is a difficult technical problem but even with good packing a great amount of care must be given to the column end fittings and the inlet and outlet connection to keep dispersion to a minimum. Some state of the art systems are now 'chip' based and may use no particles at all. LC columns, in general, achieve their separation by exploiting the different intermolecular forces between the solute and the stationary phase and those between the solute and the mobile phase. The column will retain those substances that interact more strongly with the stationary phase than those that interact more strongly with the mobile phase. The main consideration with HPLC is the much wider variety of solvents and packing materials that can be utilized because of the much lower quantities of both which are required. In particular very expensive optically pure compounds can be used to make Chiral HPLC stationary phases and may even be used as (disposable) HPLC solvents.

- Considered the “heart of the chromatograph” the column’s stationary phase separates the sample components of interest using various physical and chemical parameters.
- The small particles inside the column are what cause the high backpressure at normal flow rates.
- The pump must push hard to move the mobile phase through the column and this resistance causes a high pressure within the chromatograph

4.Detectors

A large number of HPLC detectors have been developed based on a variety of different sensing principles. The four dominant detectors used in LC analysis are the *UV detector* (fixed and variable wavelength), the *Diode Array Detector,* the *electrical conductivity detector*, the *fluorescence detector* and the *refractive index detector*. These detectors are employed in over

95% of allLCanalytical applications. These five detectors are described has been detailed. Detector sensitivities and detector linearity will, however, be given for each of the four detectors.

- It is used to detect the individual molecules that come out (elute) from the column.
- A detector serves to measure the amount of those molecules so that the chemist can quantitatively analyze the sample components.
- The detector provides an output to a recorder or computer that result in the liquid chromatogram (i.e., the graph of the detector response).

(i) The UV Detector

The UV detector is the most popular HPLC detector usingsingle wavelength. Although the UV detector has some definite limitations (particularly for the detection of non-polar solutes that do not possess a UV chromophores) it has the best combination of sensitivity, linearity, versatility and reliability of all the HPLC detectors so far developed.

Most compounds adsorb UV light in the range of 200-350Å including all substances having one or more double bonds and all substances that have unshared (non-bonded) electrons; *e.g.* all olefins, all aromatics and all substances containing >CO, >CS, -N=O and -N≡N-groups. The relationship between the intensity of UV light transmitted through the detector cell and solute concentration is given by "Beers' Law,

$$I_T = I_o e^{-kLc}$$

where, (I_o) is the intensity of the light entering the cell,(I_T) is the intensity of the transmitted light,(L) is the path length of the cell,(c) is the concentration of the solute,(k) is the molar extinction coefficient of the solute for thespecific wavelength of the UV light.

$$LnI_T = InI_o - kLc \text{or} I_T = I_o e 10^{-k'Lc}$$

where (k') is the molar extinction coefficient of the solute.

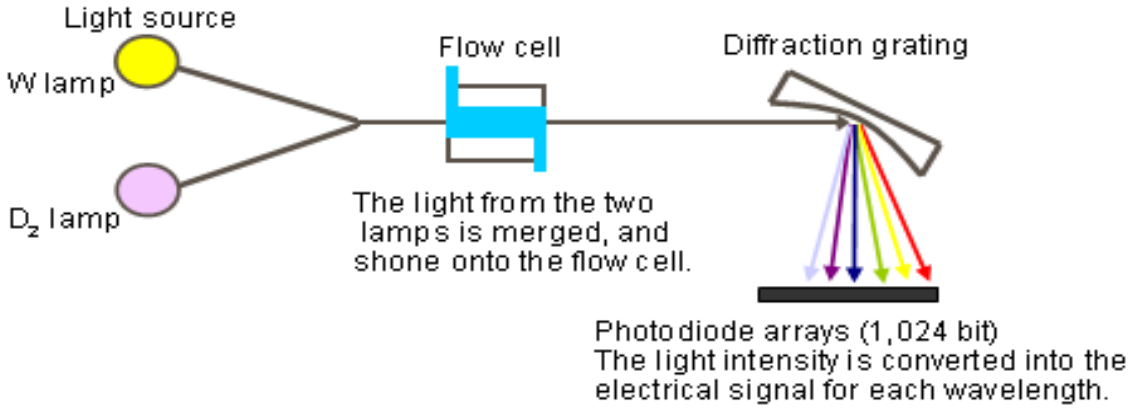
$$\frac{\partial\left(Ln\frac{I_T}{I_o}\right)}{\partial_c} = -kL$$

It is seen that there are two factors that control the detector sensitivity, the magnitude of the extinction coefficient of the solute being detected (which will depend on the wavelength of the UV light that is used) and the path length of the light passing through the cell. Thus, although the minimum detectable concentration can be changed by selecting a light source of different wavelength, the cell length cannot be increased indefinitely to provide higher sensitivity as long cells will provide excessive peak dispersion with consequent loss of column resolution. It follows, that the optimum detector cell design involves the determination of the cell length that will provide the maximum sensitivity and at the same time constrain detector dispersion to a minimum so that there is minimum loss in resolution.

(ii)The Diode Array Detector

Light from a broad emission source such as a deuterium lamp is collimated by an achromatic lens system so that the total light passes through the detector cell onto a holographic grating. In this way the sample is subjected to light of all wavelengths generated by the lamp. The dispersed

light from the grating is allowed to fall on to a diode array. The array may contain many hundreds of diodes and the output from each diode is regularly sampled by a computer stored on a hard disc. At the end of the run, the output from any diode can be selected and a chromatogram produced employing the UV wavelength that was falling on that particular diode.



Diagrammatic representation of DAD optics

Most instruments will permit the monitoring of a least one diode in real time so that the chromatogram can be followed as the separation develops. This system is ideal in that by noting the time of a particular peak, a spectrum of the solute can be obtained by recalling from memory the output of all the diodes at that particular time. This gives directly the spectrum of the solute, *i.e.*, a curve relating adsorption against wavelength.

The performances of both types of multi-wavelength detectors are very similar and typical values for their more important specifications are as follows.

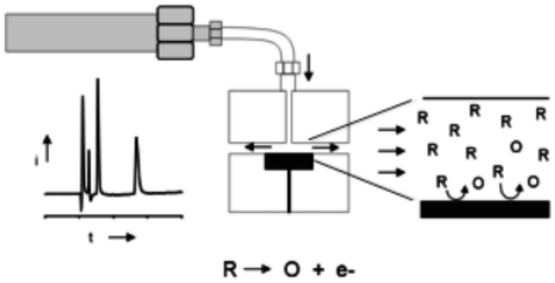
Typical Specifications for a Multi-Wavelength UV Detector

Sensitivity	1 x 10^{-7}g/ml
Linear Dynamic Range	5 x 10^{-7} to 5 x 10^{-4} g/ml
Response Index	0.97 - 1.03

(iii)The Electrochemical Detector

The electrochemical detector responds to substances that are either oxidizable or reducible and the electrical output is an electron flow generated by a reaction that takes place at the surfaceof the electrodes. If the reaction proceeds to completion (exhausting all the reactant) the current becomes zero and the total charge generated will be proportional to the total mass of material that has been reacted.

This process is called *coulometric* detection. If, however, the mobile phase is flowing past the electrodes, the reacting solute will be continuously replaced as the peak passes through the detector. All the time there is solute present between the electrodes, a current will be maintained, albeit varying in magnitude. Until relatively recently, this procedure was that most common employed in electrochemical detection and is called *amperometric* detection.



The electrochemical detector requires three electrodes, the working electrode (where the oxidation or reduction takes place), the auxiliary electrode and the reference electrode (which compensates for any changes in the background conductivity of the mobile phase).

The processes taking place at the electrode surface can be very complex; nevertheless, the dominant reaction can be broadly described as follows. At the actual electrode surface the reaction is extremely rapid and proceeds almost to completion. This results in the layer close to the electrode being virtually depleted of reactant. As a consequence, a concentration gradient is established between the electrode surface and the bulk of the solution. This concentration gradient causes solute to diffuse into the depleted zone at a rate proportional to the solute concentration in the bulk of the mobile phase. Thus, the current generated at the electrode surface will be determined by the rate at which the solute reaches the electrode and consequently, as the process is diffusion controlled, will depend on solute concentration and the magnitude of solute diffusivity.

The response of the detector or current (i) is described by the following equation,

$$i = nFAK_T cu^a$$

where

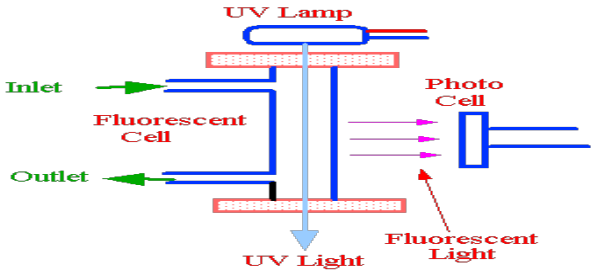
(n) is the number of electrons per molecule involved in the reaction,
(F) is the Faraday Constant,
(A) is the area of the working electrode,
(KT) is the limiting Mass Transfer Coefficient,
(c) is the solute concentration,
(u) is the linear velocity of the mobile phase over the surface of the electrode &
(a) is a constant usually taking a value between 1/3 and 1/2.

It is seen that the current (i) (and, thus, the sensitivity), can be raised by increasing the electrode area, increasing the transfer coefficient or increasing the velocity of the mobile phase past the electrodes. It would appear that increasing the electrode area would be the easiest; however, increasing the electrode surface area while maintaining an amperometric response also increases the noise, often to such an extent that there is an overall *reduction* in detector sensitivity. It is shown that under certain conditions a *reduction* in the sensor size produces a significant increase in signal-to-noise and, thus an increase in sensitivity. Higher flow rates will also increase the rate of solute transfer and the sensitivity which would be an added advantage to miniaturization. However, the sensor will be very flow sensitive and thus the flow rate must be

kept very constant and the detector would not be amenable to flow programming development. Miniaturization would also reduce peak dispersion in the sensor.

(iv)The Fluorescence Detector

The fluorescence detector is one of the most sensitive LC detectors and for this reason is often used for trace analysis. Unfortunately, although the detector is very sensitive, its response is only linear over a relatively limited concentration range. In fact, the response of the detector can only be assumed to be linear over a concentration range of two orders of magnitude. Unfortunately, the majority of substances do not naturally fluoresce which is a serious disadvantage to this type of detector. It follows, that in many instances fluorescent derivatives must be synthesized to detect the substances of interest. There are a number of regents that have been developed specifically for this purpose but derivatizing procedures should be thoroughly followed. A diagram of the Fluorescence Detector is shown below with typical specification



The Fluorescence Detector

In its simplest form, light from a fixed wavelength UV lamp passes through a cell, through which the column eluent flows and acts as the excitation source. Any fluorescent light that is emitted is sensed by a photo electric cell positioned normal to the direction of exciting UV light. The photo cell senses fluorescent light of all wavelengths but the wavelength of the excitation light can only be changed by use of an alternative lamp. This simple type of fluorescence detector was the first to be developed, it is relatively inexpensive and for certain compounds can be extremely sensitive. Typical specifications for a fluorescence detector are as follows:-

Typical Specifications for a Fluorescence Detector

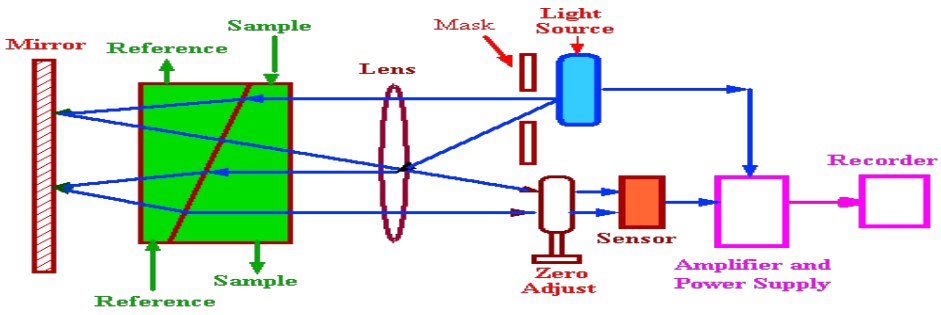
Sensitivity (Anthracene)	1x 10^{-9} g/ml
Linear Dynamic Range	1 x 10^{-9} to 5 x 10^{-6} g/ml
Response Index	0.96 - 1.04

A more elaborate form of fluorescence detector uses a monochromator to select the excitation wavelength and a second monochromator to select the wavelength of the fluorescent light. This instrument gives the maximum versatility and allows the maximum sensitivity to be realized for any type of solute. The system can also provide fluorescence spectra by arresting the flow of mobile phase when the solute resides in the detecting cell and scanning the fluorescent light.

(v)The Refractive Index Detector

The refractive index detector is one of the least sensitive HPLC detectors. It is very sensitive to changes in ambient temperature, pressure changes, flow-rate changes and cannot be used for gradient elution. Despite these many disadvantages, this detector is extremely useful for

detecting those compounds that are nonionic, do not adsorb in the UV, and do not fluoresce. There are many optical systems used in refractive index detectors (9) but one of the most common is the differential refractive index detector shown diagramatically in below



The Refractive Index Detector

The refractometer shown in figure monitors the deflection of a light beam caused by the refractive index difference between the contents of the sample cell and that of the reference cell. A beam of light (usually from an incandescent lamp) passes through an optical mask that confines the beam to the region of the cell. The lens collimates the light beam which then passes through both the sample and reference cells to a plane mirror. The mirror reflects the beam back through the sample and reference cells to a lens which focuses it onto a photo cell. The location of the beam, rather than its intensity, is determined by the angular deflection of the beam resulting from the refractive index difference between the contents of the two cells. As the beam changes its position of focus on the photoelectric cell, the output changes and the difference signal is electronically modified to provide a signal proportional to the concentration of solute in the sample cell.

The refractive index detector is often a 'choice of last resort' and is selected for those applications where, all other detectors are inappropriate or impractical. However, the detector has one particular and unique area of application and that is in the separation and analysis of polymers. For those polymers that contain more than ten monomer units, the refractive index is directly proportional to the concentration of the polymer and is practically independent of the molecular weight. A quantitative analysis of a polymer mixture can, therefore, be obtained by the simple normalization of the peak areas in the chromatogram (there being no need for the use of individual response factors). Some typical specifications for the refractive index detector are as follows:-

Typical Specifications for a Refractive Index Detector

Sensitivity (benzene)	1x 10^{-6} g/ml
Linear Dynamic Range	1 x 10^{-6} to 1 x 10^{-4} g/ml
Response Index	0.97 - 1.03

A typical application of the RI detector is for carbohydrate analysis. Carbohydrates do not adsorb in the UV, do not ionize and although fluorescent derivatives can be made, the procedure is tedious and time consuming. An example of such an application is shown in figure 28 by the separation of the products of cyclodextrin hydrolysis.

5. Computer/Display

A device that records the electrical response of a detector on a computer screen in the form of a chromatogram. Advanced data recording systems also perform calculations using sophisticated algorithms, *e.g.,* to integrate peak areas, subtract baselines, match spectra, quantitate components, and identify unknowns by comparison to standard libraries.

Frequently called the data system, the computer not only controls all the modules of the HPLC instrument but it takes the signal from the detector and uses it to determine the time of elution (retention time) of the sample components (qualitative analysis) and the amount of sample (quantitative analysis).

Chromatogram

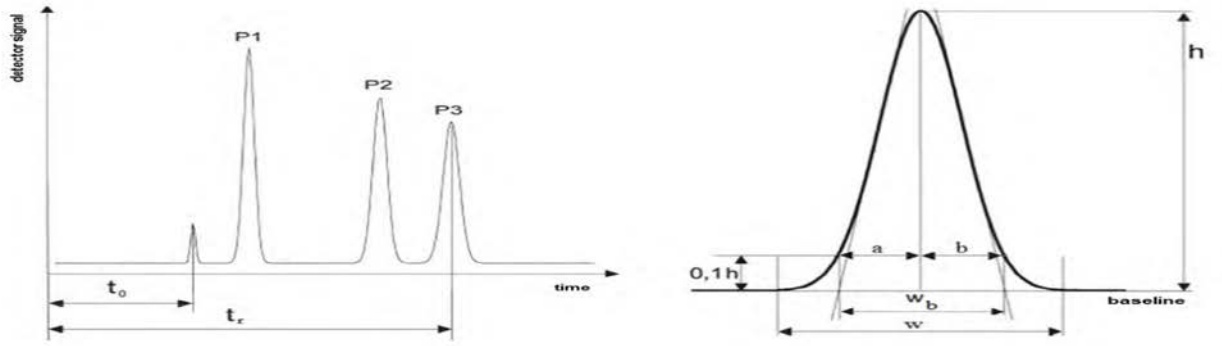
A graphical representation of detector response as a measure of the concentration of the analyte in the eluent versus time is called Chromatogram.

Baseline

The portion of the chromatogram recording the detector response when only the mobile phase emerges from the column.

Delay time (t_0)

The delay time refers to the time which is required for a non-retarded compound to be transported from the injection site to the detector unit (where the compound is recorded). During this time, all sample molecules are exclusively located in the mobile phase. In general, all sample molecules share the same delay time. The separation is caused by differing adherence of the substances with the stationary phase.



Retention time (t_r)

The retention time refers to the time which is required for a compound from the moment of injection until the moment of detection. Accordingly, it represents the time the analyte is in the mobile and stationary phase. The retention time is substance-specific and should always provide the same values under the same conditions.

Peak width (w)

The peak width covers the period from the beginning of the signal slope until reaching the baseline after repeated drop in the detector signal.

Tailing factor (T)

Practically, symmetric peaks are very rare. In a chromatogram they often show some degree of tailing. Peak tailing is measured by the tailing factor T.This factor describes the peak asymmetry, i.e. to which extent the shape is approximated to the perfectly symmetric Gaussian curve. T = 1 represents a symmetrical peak. For T > 1 the peak profile is named tailing. For T < 1 the peak profile is named fronting.
The tailing factor is measured as: T=b/a
a represents the width of the front half of the peak, b is the width of the back half of the peak.
The values are measured at 10 % of the peak height from the leading or trailing edge of the peak to a line dropped perpendicularly from the peak apex.

Resolution[Rs, see Selectivity]

The separation of two peaks, expressed as the difference in their corresponding retention times, divided by their average peak width at the baseline. R**s** = 1.25 indicates that two peaks of equal width are just separated at the baseline. When R**s** = 0.6, the only visual indication of the presence of two peaks on a chromatogram is a small notch near the peak apex. Higher efficiency columns produce narrower peaks and improve resolution for difficult separations; however, *resolution* increases by only the *square root of N*. The most powerful method of increasing resolution is to increase *selectivity* by altering the mobile/stationary phase combination used for the chromatographic separation [see section on Chemical Separation Power].

Retention Factor[k]
A measure of the time the sample component resides in the stationary phase relative to the time it resides in the mobile phase; it expresses how much longer a sample component is retarded by the stationary phase than it would take to travel through the column with the velocity of the mobile phase. Mathematically, it is the ratio of the adjusted retention time [volume] and the hold-up time [volume]: k = t**R**'/t**M** [see Retention Time and Selectivity].

Selectivity [Separation Factor, σ]
A term used to describe the magnitude of the difference between the relative thermodynamic affinities of a pair of analytes for the specified mobile and stationary phases that comprise the separation system. The proper term is *separation factor* [σ]. It equals the ratio of retention factors, k2/k1 = 1, then both is always ≥ 1. If σ [see Retention Factor]; by definition, σ peaks co-elute, and no separation is obtained. It is important in preparative chromatography to maximize α for highest sample loadability and throughput.

Sensitivity [S]
The signal output per unit concentration or unit mass of a substance in the mobile phase entering the detector, *e.g.,* the slope of a linear calibration curve [see Detector]. For concentration-sensitive detectors [*e.g.,* UV/VIS absorbance], sensitivity is the ratio of peak height to analyte

concentration in the peak. For mass-flow-sensitive detectors, it is the ratio of peak height to unit mass. If sensitivity is to be a unique performance characteristic, it must depend only on the chemical measurement process, not upon scale factors. The ability to detect [*qualify*] or measure [*quantify*] an analyte is governed by many instrumental and chemical factors. Well-resolved peaks [maximum selectivity] eluting from high efficiency columns [narrow peak width with good symmetry for maximum peak height] as well as good detector sensitivity and specificity are ideal. Both the separation system interference and electronic component noise should also be minimized to achieve maximum sensitivity.

Environmental Applications of HPLC

- Phenols in Drinking Water. Identification of diphenhydramine in sediment samples.
- Biomonitoring of PAH pollution in high-altitude mountain lakes through the analysis of fish bile. Estrogens in coastal waters - The sewage source.
- Toxicity of tetracyclines and tetracycline degradation products to environmentally relevant bacteria. Assessment of TNT toxicity in sediment.

Demo: Analysis of PAHs on HPLC

Mr.GNV Satyanarayana

Technical officer
Analytical Chemistry Section, Regulatory Toxicology Group
CSIR-Indian Institute of Toxicology Research, Lucknow

Online demonstration through Video will organised

Method standardization of multiclass pesticides in environmental samples

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India is an agriculture based country. The five basic constituents i.e. soil, water, sunlight, air and space are the creativity factors of the whole world and the activities. “If we seed the Agriculture, it will feed the Nation”. The agricultural crops are the baby of us. So, it is our moral responsibility to protect the crops from any type of injury rather attention to be given towards crop health and soil. Pesticides are an inevitable key tool in maintaining sustainable food-grain production in modern agricultural farming system. Pesticides are further divided as per use to control pests e.g. Insecticide, fungicide, herbicide, rodenticide, etc., etc. Again based on chemical nature it may be Organochlorine, Organophosphorus, Organocarbamates, Synthetic pyrethroids, Neo-nicotinoids, Pyrazole, Carbamates/Urea derivatives herbicides, PhenoxyAlkanoic Acid, Triazole and so many. Various mode of action of different agro-chemicals are also available in the market whose use pattern could help us in minimizing development of resistance. Now, from the chemical point of view all the pesticides are toxic and this is the intrinsic property of the chemical molecule by nature. Pesticides find its application not only to the agricultural crop but also other sectors viz. home / industrial / institution / water treatment plants / vector controlling programme / **v**eterinarian / wood preservative, etc. as a preventive/protective measure. As a result pesticides may do leave residues in different raw agricultural produce, processed foods and various compartments of the environment. Automatically, it will strike our mind regarding the fate of pesticides in the food and environment. Thus, coupling of the two factors i.e. application of toxic chemicals on one hand and consequently the presence of its residues in different matrices lead to the issue of measurement. Now, when the question of measurement comes, we search for residue chemists or analytical chemists. Here begins the challenging role of chemists as chemical analysis is a vital component of every quality management system. Determination of organic contaminants in food needs to continually revised and explore new technologies to enhance sensitivity, selectivity, separation, interpretation, and adaptability of methodology are decisive to analytical chemists and instrumentalists who are challenged with the identification and quantitation of new and already recognized xenobiotics of health concern. Food quality and food safety are the biggest issue of interest to the public resulting stringent legal requirements of safety and labeling. The assessment of food safety is a scientific exercise performed by scientists and consists of hazard identification, hazard characterization, exposure assessment and risk characterization. An important prerequisite for performing risk assessment adequately is the presence of residue data generated by reliable and fit-for-purpose analytical methods to estimate the level of exposure and intake of the consumer to contaminants and residues. Hence, the accuracy of risk assessment will benefit from the availability of comprehensive quantitative monitoring and consumption data. Thus, a strong additional demand for adequate methods of analysis has occurred.

The analysis of agricultural products for pesticide residues is of importance worldwide. Almost every country either imports or exports food. Most countries do both. The allowable residue requirements for an exporting country are often not the same as the importing country. There is an obvious need for test procedures that provide unambiguous results in the low ppb range for a wide variety of agricultural product matrices. The analytical methods used for routine analysis must be properly validated and should be applied following quality control criteria as well as following statistical rules to assess the quality of the results. At the same time, the method of analysis must be very simple, fast, and robust so that requirements for personnel training, time spent per sample, and maintenance of the equipment can be minimized. The results must be confirmed by mass spectrometric (MS) detection due to the high risk of false positives instead of using other common selective detectors (ECD, NPD, FPD, TID, etc) with gas chromatography (GC) or UV-VIS, Photo-Diode Array (PDA) detector in HPLC. There are various multi-residue methods for determining multiclass pesticide residues but majority of these are time consuming or tedious and are applied with difficulty in routine analysis in a laboratory designed to control food quality. While analyzing large number of samples for determining multiclass pesticide residues one has to inject samples in different detectors for identification and quantification which implies that the instrumental step is doubled or tripled and very less number of samples can be analyzed per day. Moreover, these types of methods are laborious, expensive and increase the uncertainty of the final results. The selectivity advantage of GC/MS/MS or LC-MS/MS allows an analyst to estimate and identify ultra trace level components in the most difficult matrices. In the present day, Mass Spectrometry is used as one of the most versatile analytical techniques in the identification, confirmation and quantification of organic molecules in the environmental field. The present discussion will be limited to the **method validation and instrument performance verification for method standardization of multiclass pesticides in environmental samples.**

Validation and Verification of Multi-class Pesticide Residue Analytical Method

Any work carried out in a systematic particular way is known as **method**. The method must be reliable, effective and scientific for data analysis. And to make objects or activities of the same type have the same features or qualities is known as **standardization**.

Why chemists do required method standardization of multi-class pesticide?

The answer is that the Analytical methods are used to generate the data for estimating dietary exposure assessments, to establish Maximum Residue Levels (MRLs), and to determine processing factors. Analytical methods are also used in enforcement of any statutory MRLs that may be established. Methods apply to all pesticides used on edible crops/livestock and subsequent produce and processed food products, and for products (e.g. meat, milk, eggs) from animals that may consume treated crops and environmental samples. Additionally, analytical methods are also needed for conducting storage stability studies.

The **practical approach** for method standardization may be broadly divided into two heads: a) Laboratory bench work b) Instrumental analysis. Therefore, before developing a residue

analytical method of multi-class pesticide in any components of the environment, a chemist must gather preliminary knowledge on the following points:

1. **Matrices:** What type of substrate you want to analyze? Is it the raw agricultural produce or processed foods or environmental samples or what else? Try to know the nutritional index of food products. This will help you in selecting some specific chemicals e.g. PSA, GCB, C-18 cartridges, etc. for removal of those unwanted matrix components. For soil, sediment the information on physico-chemical properties are important for choosing silica-gel or alumina or florisil or graphitized carbon in order to separate co-extracted matrix.

2. **Pesticides:** See the solubility and storage stability from the literature which will help in selecting the extraction solvent from the matrices. As the case is for multi-class pesticide it is definite that all will bear not the same properties. Try to group in acidic, basic and neutral. Also you can group into Organochlorine, Organophosphorus, Organocarbamates, Synthetic pyrethroids, Neo-nicotinoids etc. or insecticides, fungicides, herbicides, etc. depends on the objective of your work.

3. **Identify the GC or LC amenable pesticides.**
   It is important to note that the methods include the analytes in accordance with the residue definition for the particular pesticide. The residue definition used for dietary risk assessment purposes may differ from that used for MRL enforcement purposes. For MRL enforcement, the methodology applied in multi residue methods is different from country to country and strongly depends on the available equipment and the capability of the individual laboratory.

After completion of the above three points, get ready for method standardization. The method standardization involves: Preparation of mother stock solution of individual analytical pesticide standard, select a method of analysis from literature review or sketch the in-house method, validate the method following validation criteria and Instrumental performance verification.

The steps for method standardization:

A. Preparation of mother stock solution of individual analytical pesticide standard

Preparation of mother stock solutions is of utmost importance in pesticide residue analysis. Any mistake in the preparation of stock solutions reflects in the final results and the entire exercise will be futile.

The SOP for the preparation of mother stock solutions is as follows:

1) Check the Certificate of Analysis and Date of Expiry of the CRM
2) TakeouttheCRMbottlefromthedeepfreezerandwaitfor1houruntiltheCRM attains room temperature.
3) Weigh the empty calibrated volumetric flask/graduated tube (100or 50mL) using a calibrated analytical balance and tare the weight.

4) Transfer carefully 20-30mg of CRM with the help of spatula (in case of solid) and using capillary tubes (in case of Viscous /semisolid) into the volumetric flask and note the weight.
5) Dissolve the CRM using approximately 2-3mL of analytical grade acetone for gas chromatographic (GC) analysis. For Liquid Chromatographic (LC) analysis, use acetonitrile or methanol.
6) Makeup the volume with analytical grade hexane:toluene(1:1) for GC and for LC, use acetonitrile or methanol.
7) Label the flask with name of the standard, concentration of the stock solution (ppm), solvent used, date of preparation, volume and weight.

Calculation: Calculate the final concentration of the mother stock solution using the formula:

$$\text{Concentration (ppm)} = \frac{\text{Wt ofCRM (mg)} \times 10^3 \times \text{PurityofCRM (\%)}}{\text{Final Volume(mL)} \times 100}$$

Note: ppm= µg/mL or µg/g or mg/Kg or mg/L

$mg \times 10^3 = \mu g$ $g \times 10^6 = \mu g$

The records of the stock of CRM along with the weight of mother stock solution are maintained in the Register for future use.

Working standard solutions

1) Mother Stock solutions are normally diluted to prepare100ppm intermediate working standards using the formula N1V1=N2V2 with the solvents viz.acetone, toluene, hexane for GC compounds and acetonitrile /methanol for LC compounds.
2) Label the flask with name of the standard, concentration of the stock solution, solvent used, date of preparation and volume. The records of working standard are maintained in register.
3)Further subsequent intermediate working standards(individual/mixture) are prepared by serial dilution technique as per laboratory requirements from time to time using the formula N1V1 =N2V2 with the solvents viz.acetone, toluene, hexane for GC compounds and acetonitrile/methanol for LC compounds.

Preparation of multi-class pesticide mixture solution (10 and 1 ppm):
Say for example: You have 100 ppm individual standard solution of 50 compounds. Make five clusters of 10 compounds. Take five 10 ml graduated tube and keep it in a test tube rack. Using micro pipette take out 1 ml from 100 ppm and transfer to 10 ml graduated tube. Like this add 9 others compounds. This makes 10 ppm mixture of ten compounds. Similarly, for the rest cluster. So, now you have **10 ppm** multi-class mixture of 50 compounds in five graduated tube.

Multi-class mixture of working standard (1 ppm) of 50 compounds:
Take one 10 ml graduated tube and keep it in a test tube rack. Using micro pipette take out 1 ml from each **10 ppm** multi-class pesticide mixture prepared. So altogether you have taken 5 ml

and the rest 5 ml make up the volume with desired solvent. This gives the mixture of 50 compounds of 1 ppm in one test tube. Please note that working standard should not be use more than 7 days.

How to know the spiking concentration level in the matrix?
Say for e.g. You have 50 ppm standard solution. i.e. 50 µg/mL, it means if you take 1 mL from 50 ppm solution, it contains 50 µg. And if you add this 1 mL solution to 25 g of vegetable, then the concentration of pesticide in vegetable will be 50/25=2 ppm.

Precaution: Care should be taken to see that the working standard solutions donot get concentrated due to repeated opening of the bottle. The stability of the working solutions must be monitored at regular intervals. After taking required volume from mother stock solution for preparation of intermediate stock, note the weight of the mother stock solution and note the same in Register before keeping it back in refrigerator. This helps making sure that the weight is made-up before further use, if any loss or evaporation during storage occurred.

B. Selection of a method of analysis from literature reviews or sketches the in-house method and give a trial of run of the mixture standard.

Care should be given on the minimum concentration to be reported and which instrument it is to be analyzed. Then prepare the multi-class pesticide (MCP) mixture of minimum concentration and inject in the instrument. See the S/N ratio > 10. If it is OK you can go ahead after checking all the validation parameters and if required little bit modification may be done. Practically the method is developed keeping in mind the reporting level. For that modification may be done in sample weight or choosing extracting solvent or final volume make up.

C. Validation of Residue Analytical Method and Acceptable Criteria

The objective of analytical method validation is to demonstrate that the procedure, when correctly applied, produces results that are fit for purpose. The procedures to be carried out to validate the analytical methods included as part of registration. During method development and validation the following objectives is to be fulfilled.

The method(s) should: have the ability to determine all of the likely analytes that may be included in the residue definition (both for risk assessment and enforcement); be sufficiently selective so that interfering substances never exceed 30% of the limit of analytical quantitation (LOQ); demonstrate acceptable recovery and repeatability; cover all crops, animals, and feed items being treated. If significant residues occur, cover processing fractions and drinking water; and cover all edible animal commodities if animals are likely to consume treated crops. However, some regulatory authorities will establish maximum residue limits for edible animal commodities for trade purposes, although no residues are expected in those commodities. Enforcement methods are therefore required to demonstrate appropriate limits of quantitation and to establish MRLs at LOQ.

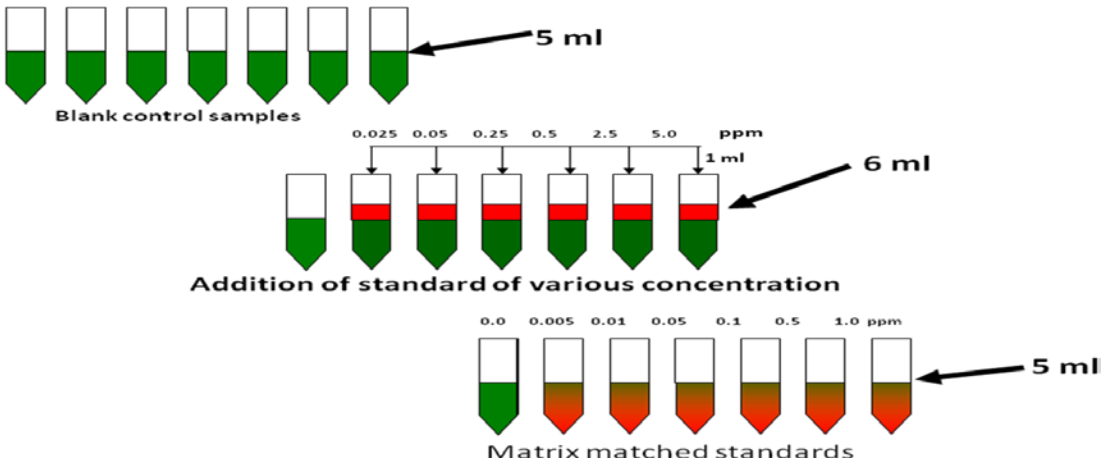
Validation parameters definition on residue analytical methods

To be fit for the intended purpose, the method should meet standards for certain validation parameters. The validation characteristics for residue analytical methods that should be considered are: linearity, accuracy, recovery, selectivity (specificity), calibration, precision (repeatability, reproducibility), limit of detection (LOD), and limit of quantitation (LOQ). These parameters are defined below:

Linearity: At least 5-point calibration curve will be generated with the mixture of analytical standard of pesticides in scope at different concentrations (e.g.1.0, 0.5, 0.1, 0.05 and 0.01 mg/kg) dissolved in pure solvent for estimating detection limit. The calibration curve is done by plotting detector response (x) vs concentration (c) and a regression analysis on the calibration curve will be obtained by using the inbuilt software of the instrument.

Matrix Match Standard Calibration curve: To eliminate the matrix interferences or matrix enhancement effect if seen, Matrix Match Calibration standard is more useful than pure solvent calibration curve in the interpretation of data and could lead to more accurate recovery results. Matrix Match calibration standard solution is prepared in the following way:

I. Prepare at least five different concentrations (viz. 1.0, 0.5, 0.1, 0.05 and 0.01 mg/kg or as per requirement) of mixture of pesticide in scope with pure solvent.
II. Prepare at least six (6) blank control samples of the matrix in scope following the Analytical Test Method for residue determination of the matrix concerned to be validated.
III. Keep one blank control sample separately.
IV. Add 1 ml of different concentration of mixture of pesticide solution to the rest five blank control samples separately.
V. Evaporate to dryness.
VI. Make up volume with the desired solvent as per the methodology.
VII. Matrix match standard solution of mixture of pesticides of different concentration thus obtained.
VIII. Inject different concentration of matrix match standard into chromatographic instrument.
IX. Note down its area.
X. Calculate the values of slope (m), intercept (i) and r^2 for a number of standards using regression analysis.
XI. Thus, Matrix Match Standard calibration curve is generated. The schematic diagram is presented below



Preparation of Matrix Match Standard Calibration curve

Alternatively, carry out the recovery experiment at different concentration level as chosen for linearity establishment and set it as Matrix match calibration curve. This will give you more and more accurate results of recovery study.

Selectivity (Specificity): Selectivity refers to the extent to which the method can be used to determine particular analytes in mixtures or matrices without interferences from other components of similar behaviour. Some regulatory authorities use the term specificity to refer to selectivity.

Sensitivity: The sensitivity of the method should be assessed by the limit of detection (LOD) and limit of quantification (LOQ).Limit of detection is defined as the smallest amount of an analyte that can be reliably detected or differentiated from the background for a particular matrix (by a specific method). Limit of quantification (also known as limit of determination) isdefined as the lowest concentration tested, at which an acceptable mean recovery (70-110%), with an acceptable RSD (< 20%), and S/N ratio 10 or more is obtained. With most methods and determination systems, the LOQ has no fixed value.

For most modern analytical methods, the LOD may be divided into two components, instrumental detection limit (IDL) and method detection limit (MDL). IDL may be defined as the smallest amount of an analyte that can be reliably detected or differentiated from the background on an instrument. The IDL is dependent on various factors such as sensitivity of the detector for the analyte of interest and electronic and detector (instrumental) noise of various origins, The method detection limit (MDL) is a term that should be applied to extraction and analysis methods developed for the analysis of specific analytes within a matrix. The MDL can be defined as the smallest amount of an analyte that can be reliably detected or differentiated from the background for a particular matrix (by a specific method). All matrix interference must be taken into consideration when determining the MDL.

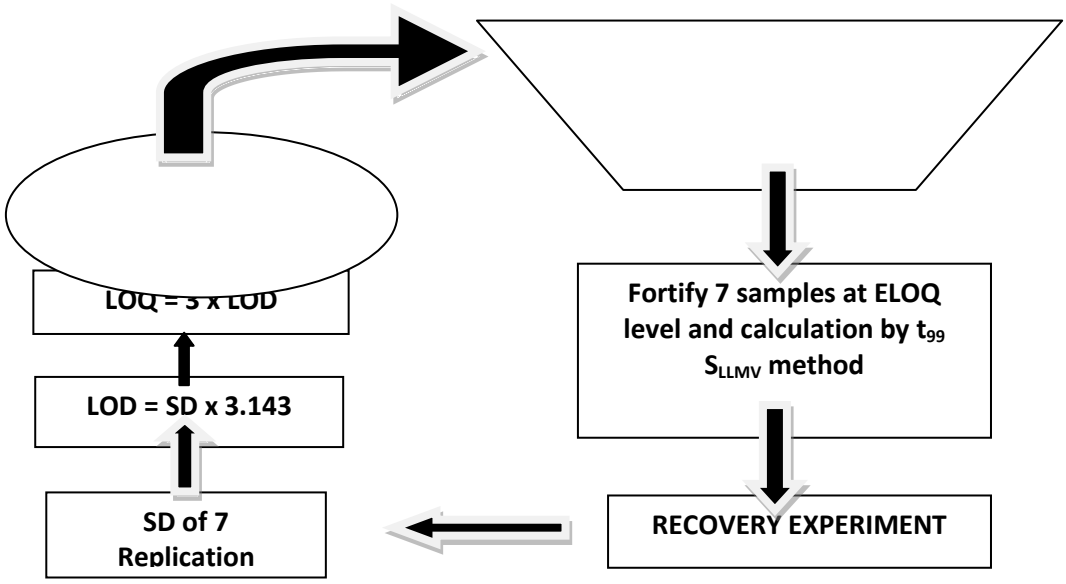
Similarly, the LOQ may be divided into two components, instrumental quantification limit (IQL) and method quantification limit (MQL). IQL may be defined as the smallest amount of an analyte that can be reliably quantified by the instrument and MQL can be defined as the smallest amount

of an analyte that can be reliably quantified with a certain degree of reliability within a particular matrix (by a specific method).

Basis of establishing limit of detection (LOD) and limit of quantification (LOQ):

A Two – step approach method as recommended by US EPA may be followed for estimating Limit of detection (LOD) and Limit of quantification (LOQ). The methods are (i) Root Mean Square Error (RMSE) method and (ii) The $t_{99}S_{LLMV}$ method. Schematically shown below:

Basis of Establishing LOD and LOQ by 2 Step Approach Method



CALCULATION OF IDL, IQL AND ELOQ in Excel Sheet

Pesticide concentration [C] in ppm	Instrumental detector response [X]	Predicted response [Xp] = [slope (m) x C] + intercept (i)] = 984.17 x C+3.1872	Error(E) = [Xp – X]	$(Error)^2$	$\Sigma E^2/n-2$	RMSE= $\sqrt{\Sigma E^2}$ / n-2	IDL= 3 x RMSE/slope (m)	IQL= 10 x RMSE/slope (m)	ELOQ= (IQL / dilution factor) x (100/recovery %) = (IQL/1) x (100/85)
1.0	**981.8557**	**987.3572**	**5.5015**	**30.2665**	**64.0374**	**8.0023**	**0.0244**	**0.0813**	**0.0957**
0.5	**507.2210**	**495.2722**	**11.9488**	**142.7738**					
0.1	**97.5938**	**101.6042**	**4.0104**	**16.08331**					
0.05	**51.1147**	**52.3957**	**1.28103**	**1.641038**					
0.01	**11.8680**	**13.0289**	**1.16087**	**1.347619**					
				ΣE^2 = 192.1123					

CALCULATION OF LOD (MDL) AND LOQ (MQL) BY RECOVERY EXPERIMENT AT ELOQ (0.0957 PPM) LEVEL AT SEVEN REPLICATIONS

PARAMETERS	REPLICATIONS						
	R1	R2	R3	R4	R5	R6	R7
Recovery (ppm)	0.093	0.088	0.091	0.070	0.069	0.080	0.086
Recovery (%)	97.22	91.993	95.129	73.176	72.131	83.525	89.902
Mean recovery (%)	86.154						
SD	0.0097						
LOD (MDL) = 3.143 * SD	0.0306						
LOQ (MQL) = 3* LOD (MDL)	0.0919						

Recovery experiment at the level of LOQ and 10 times of LOQ:

The LOQ so determined from the above procedure provides a concentration limit above which fairly precise quantitative measurements can be done. The method selection for suitability and adaptability was carried out upon considering the performance characteristics *viz*. specificity, detection limit (LOD), quantification limit (LOQ), precision, and recovery along with cost efficiency and time factor. Recovery studies were carried out in order to establish the reliability of the analytical methods and to know the efficiency of extraction and clean up step for the present study by fortifying samples at the level of LOQ and 10 times of LOQ in triplicate. Mean recoveries for each level should be in the range 70-110 %, ideally with the mean in the range 80-100%. Control (unfortified) samples should be analyzed concurrently to determine any contamination by the analyte of interest or interferences. Recovery is the amount measured as a percentage of the amount of analyte(s) originally added to a sample of the appropriate matrix.

Repeatability:

The repeatability is expressed as relative standard deviation (RSD, %) and the number of samples (n) given. The overall RSD should be ≤20% per commodity and fortification level. RSD (%) = (SD/Mean recovery) x 100. It means the recovery experiment is to be carried out with the prescribed methodology on identical test material in the same laboratory by the same operator using the same equipment within short intervals of time (7-15 days).

Reproducibility

It is defined as a validation of the repeatability of recovery experiment from representative matrices at representative levels by other laboratory. Within-laboratory or intra-laboratory reproducibility or single-laboratory reproducibility (run effect) contributes to day-to-day variations in the analytical system due to changes of analyst, batches of reagents, recalibration of instruments and laboratory environment (e.g. temperature changes).

Confirmation:

The techniques that are considered acceptable confirmatory techniques: GC-MS or LC-MS is used in routine analysis of residue samples, 2 ions are sufficient for identification/quantification.

Quantification:

The amount of residues in ppm (mg/kg) was calculated as follows:

Residues in mg/kg =

$$\frac{\text{Sample Area} \times \textbf{ng} \text{ of standard injected} \times \text{Total volume of the sample (mL)}}{\text{Standard area} \times \text{Weight of the sample (g)} \times \mu\text{L of sample injected}} \times \text{RF}$$

ng of standard injected = concentration of standard (ppm) x µL of standard injected, RF = Recovery factor (Rec. percentage / 100).

Validation parameters acceptable criteria or minimum performance characteristics for methods (Lab to lab varies).

Parameter	What/How	Criteria
Linearity	Through calibration curve	Residuals < ± 20%
Matrix effect	Comparison of response from solvent standards and matrix-matched	± 20 %
Reporting Limit(RL)	By definition:lowest level for which it has been demonstrated that criteria for accuracy and precision have been met	≤MRL
Specificity	The specificity of the method by definition is that the detected signal is solely due to the analyte, not another compound. It is carried out by seeing the response in reagent blank and control samples	< 30%ofRL
Accuracy	Determine average recovery for both spike levels	70-120%
Precision (RSDr)	Determine repeatability(RSDr),for both spike levels	≤20%
Precision (RSDwR)	Determine within laboratory reproducibility	≤20%
S/N ratio	Using the software of the instrument	≥ 10
Method validation performance	Satisfactory	Recovery =70-120% RSD=≤20% S/N = > 10

D. Instrument Performance Verification

This step is often neglected. In true practice it has got immense value in method validation and routine analysis. All the apparatus, equipments, instruments are used for method validation must be checked for their performance verification in a periodic manner. There must be a defined protocol in the laboratory and someone should be designated for monitoring the routine activities.

PERFORMANCE VERIFICATION PRACTICES

Intermediate check items of GC and UPLC in quarterly mode:

Test Item for Intermediate check	How	Acceptable criteria
Detector sensitivity	Use lindane&imidacloprid of five different concentration and inject into GCMS and UPLCMSMS respectively and obtain the linearity using five point calibration curve	$R^2 \geq 0.99$
Injector repeatability	Inject 7 times lindane (1 mg/kg) in GCMS and imidacloprid (0.1 mg/kg) in UPLCMSMS from the same vial and note its area & RT. Calculate RSD.	5% RSD
Injector volume precision	Inject five times lindane (1mg/kg) in GCMS and imidacloprid (0.1 mg/kg) in UPLCMSMS at three different volumes. Calculate RSD	5% RSD
Column Oven Temperature	Using externally calibrated Digital temperature	± 1 %
Flow Rate Accuracy Test	Using externally calibrated flow meter, Verify the flow Rate Accuracy of full - flow and septum purging	± 3 %
Mass Detector Calibration in GC-MS	Verify Auto tuning test (EI) Using PFTBA	Base peak should be m/z 69 along with two other peaks at m/z 219 and 502.

Miscellaneous Errors in method standardization

Dilution during Sample and Standard Preparation: Minimize the number of dilutions required to give the final dilutions of the sample and standard solutions. Each dilution step will have the potential to introduce error in the procedure.

Validation Protocol: It is highly recommended to validate an analytical procedure using some form of validation protocol. Without a validation protocol, the scientist will have a tendency to vary the experiment during the course of the validation study. Getting into the habit of creating a validation protocol will also ensure that the scientist plans before starting the experiment.

Acceptance Criteria for Validation Parameter: It is highly recommended to set acceptance criteria prior to starting validation experiments. This will provide guidance to the validating scientist on the range of acceptability of the validation results.

Documentation of Observation: It is very important to document all relevant observations during the experimental procedure. Observations are the most important information that can be used if an investigation is needed and other court cases.

Extraction procedure and estimation of Multi-class pesticide residue from Water

Filter the water sample (2.5 L) through Whatman No.42

↓

Spike 1 ml of 1.0 ppm pesticide standard mixture into Water Sample (750ml) into 1 litre separating funnel (Spike level = 0.001 ppm)

↓

Add 150g NaCl in the water sample and shake well to dissolve and add 75 ml of DCM

↓

Shake well by separating funnel and release the pressure and repeat 3 times
Place the funnel on stand for about 5 min to separate organic and aqueous layer

↓

Collect lower organic layer (DCM) in a conical flask
Repeat partitioning twice using 40 ml of DCM
Collect and combined organic layer.

↓

Add 20-30 g anhy.Na_2SO_4 to the organic layer till free flowing

↓

Concentrate on rotary vacuum /turbo-vap evaporator to about 5 ml
Repeat the concentration process twice or more with addition of hexane
until there is no smell of DCM

↓

Make final volume with 1ml of acetone for GC-ECD/confirmation by GC-MS analysis

↓

Concentrate on rotary vacuum /turbo-vap evaporator and make final volume with 1ml of acetonitrile and confirmation by LC-MS analysis

Demo: Calibration, dynamic linearity curves for quantifying pesticide residue and calculation of LOD, LOQ SE and SD

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Online demonstration through Video will organized