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HYPHENATED TECHNIQUE- A BOON TO ANALYTICAL WORLD

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ABSTRACT

Traditional analytical approaches including HPLC (High-Performance Liquid Chromatograph), GC (Gas Chromatograph), UV (Ultraviolet) detection, etc., have become insufficient to effectively handle the growing number of challenges in analyses of species- specificity and sensitivity. Modern analytical technique referred to as hyphenated techniques, originate from the traditional use of molecule or element specific detection in electrophoresis or chromatography. Currently the most common techniques for trace element speciation include a combination of separation technique coupled with a detection technique that is more sensitive. Earlier such hyphenated techniques were the coupling of separation of a special sample preparation off-line and later adding a detection technique. Presently, the hyphenated technique is developed from the coupling of a separation technique (Chromatography) and an on-line spectroscopic detection technology. Hyphenated techniques combine chromatographic and spectral methods to exploit the advantages of both. Chromatography produces pure or nearly pure fractions of chemical components in a mixture. Spectroscopy produces selective information for identification using standards or library spectra. These hyphenated techniques offer shorter analysis time, higher degree of automation, higher sample throughput, better reproducibility, reduction of contamination because it is a closed system, Enhanced combined selectivity and therefore higher degree of information. The remarkable improvements in hyphenated analytical methods over the last two decades have significantly broadened their applications in the analysis of biomaterials, especially natural products. In this article, recent advances in the applications of various hyphenated techniques, e.g., GC-MS, LC-MS, LC-FTIR, LC-NMR, CE-MS, etc. in the context of pre-isolation analyses of crude extracts or fraction from various natural sources, isolation and on-line detection of natural products, chemotaxonomic studies, chemical fingerprinting, quality control of herbal products, dereplication of natural products, and metabolomic studies are discussed with appropriate examples.

INTRODUCTION:

Hybrid Techniques (Hyphenated Techniques): A couple of decades ago, Hirschfeld introduced the term "hyphenation" to refer to the on-line combination of a separation technique and one or more spectroscopic detection techniques. This technique, developed from a marriage of a separation technique and a spectroscopic detection technique, is nowadays known as hyphenated technique (**Figure 1**).

Chromatography - Produces pure or nearly pure fractions of chemical components in a mixture.

Spectroscopy – Produces selective information for identification using standards or library spectra.

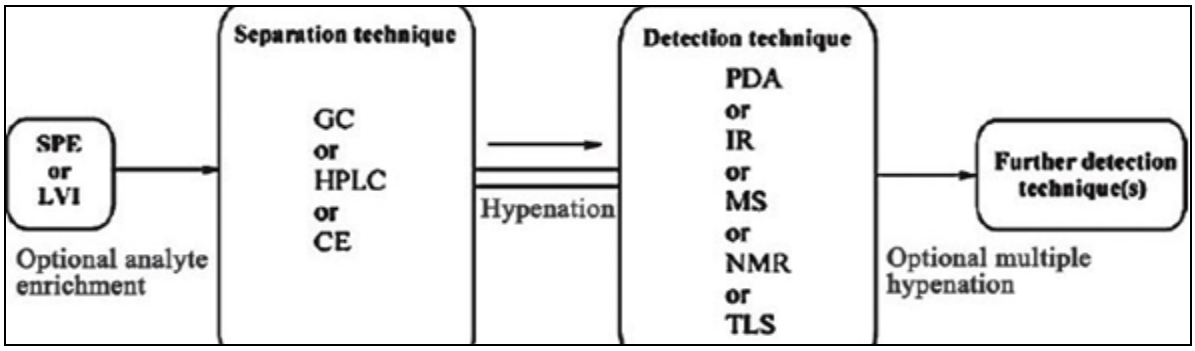

FIG. 1: HYPHENATED TECHNIQUE

In recent years, hyphenated techniques have received ever-increasing attention as the principal means to solve complex analytical problems. The power of combining separation technologies with spectroscopic techniques has been demonstrated over the years for both quantitative and qualitative analysis of unknown compounds in complex natural product extracts or fractions.

Hyphenated separation techniques refer to a combination of two (or more) techniques to detect and separate chemicals from solutions. Most often the other technique is some form of chromatography. Hyphenated techniques are widely used in chemistry and biochemistry. A slash is sometimes used instead of hyphen, especially if the name of one of the methods contains a hyphen itself.

To obtain structural information leading to the identification of the compounds present in a crude sample, liquid chromatography (LC), usually a high-performance liquid chromatography (HPLC), gas chromatography (GC), or capillary electrophoresis (CE) is linked to spectroscopic detection techniques, e.g., Fourier-transform infrared (FTIR), photodiode array (PDA) UV-vis absorbance or fluorescence emission, mass spectroscopy (MS), and nuclear magnetic resonance spectroscopy (NMR), resulting in the introduction of various modern hyphenated techniques [1].

- Gas chromatography-mass spectrometry **(GC-MS)**
- Gas chromatography-infrared spectroscopy **(GC-IR)**
- Liquid chromatography-mass spectrometry **(LC-MS),**
- Liquid chromatography-NMR spectroscopy **(LC-MNR)**
- Liquid chromatography-infrared spectroscopy **(LC-IR)**
- Capillary electrophoresis-mass spectrometry **(CE-MS)**

Advantages of Hyphenated Technique:

1. To solve complex analytical problems.
2. Shorter analysis time
3. Higher degree of automation
4. Higher sample throughput
5. Better reproducibility
6. Reduction of contamination because it is a closed system
7. Enhanced combined selectivity and therefore higher degree of information
8. Provide excellent separation efficiency as well as acquisition of on-line complementary spectro-scopic data on an LC or GC peak of interest within a complex mixture.

Types of Hyphenated Technique [2, 3, 4]:

1. **GC-MS [1]:** With MS as the preferred detection method, and single- and triplequadrupole, ion trap and time-of-flight (TOF) mass spectrometers as the instruments most frequently used, both LC-MS and GC-MS are the most popular hyphenated techniques in use today.

   GC-MS, which is a hyphenated technique developed from the coupling of GC and MS, was the first of its kind to become useful for research and development purposes. Mass spectra obtained by this hyphenated technique offer more structural information based on the interpretation of fragmentations.

   Sometimes, polar compounds, especially those with a number of hydroxyl groups, need to be derivatized for GC-MS analysis. The most common derivatization technique is the conversion of the analyte to its trimethylsilyl derivative.

Instrumentation: In GC-MS (**Figure 2**), a sample is injected into the injection port of GC device, vaporized, separated in the GC column, analyzed by MS detector, and recorded. The time elapsed between injection and elution is called ''retention time'' (tR). The equipment used for GC-MS generally consists

1. An injection port at one end of a metal column (often packed with a sand-like material to promote maximum separation)
2. A detector (MS) at the other end of the column.
3. A carrier gas (argon, helium, nitrogen, hydrogen, to name a few) propels the sample down the column. The GC separates the components of a mixture in time and
4. MS detector provides information that aids in the structural identification of each component.

The GC-MS columns can be of two types: capillary columns and macrobore and packed columns. The interface transports efficiently the effluent from the GC to MS.

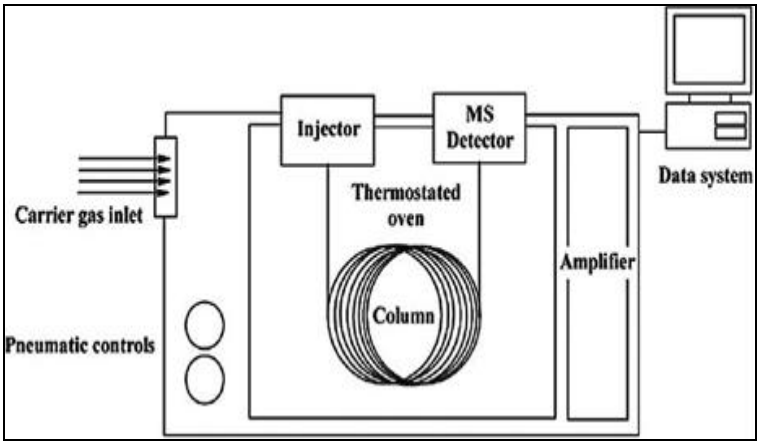

FIG. 2: GC-MS

Precautions:

1. The analyte must not condense in the interface.
2. The analyte must not decompose before entering the MS ion source.
3. The gas load entering the ion source must be within the pumping capacity of the MS.

The most extensively used interfaces for a GC-MS are electron impact ionization (EI) and chemical ionization (CI) modes. However, in modern GC-MS systems, various other types can be used that allow identification of molecular ion. For example, an orthogonal TOF mass spectrometry coupled with GC is used for confirmation of purity and identity of the components by measuring exact mass and calculating elemental composition.

Nowadays, a GC-MS is integrated with various on-line MS databases for several reference compounds with search capabilities that could be useful for spectra match for the identification of separated components.

Applications:

A. Useful for research and development purposes.

B. Identification of molecular ion.

C. Compounds that are adequately volatile, small, and stable in high temperature in GC conditions can be easily analyzed by GC-MS.

D. Used for confirmation of purity and identity of the components by measuring exact mass and calculating elemental composition.

2. **LC-IR:** The hyphenated technique developed from the marriage of an LC and the detection method infrared spectrometry (IR) or FTIR is known as LC-IR or HPLC-IR. While HPLC is one of the most powerful separation techniques available today, the IR or FTIR is a useful spectroscopic technique for the identification of organic compounds, because in the mid-IR region the structures of organic compounds have many absorption bands that are characteristic of particular functionalities, e.g., -OH, -COOH, and so on.

However, combination of HPLC and IR is difficult and the progress in this hyphenated technique is extremely slow. In addition, as a detection technique, IR is much less sensitive compared to various other detection techniques, e.g., UV and MS. The recent developments in HPLC-IR technology have incorporated two basic approaches based on interfaces applied in HPLC-IR or HPLC-FTIR.

- One is a flow-cell approach and
- Other is a solvent-elimination approach.

The approach used with the flow cell in LC-IR is similar to that used in UV-Vis and other typical HPLC detectors (**Figure 3**). In this case, absorption of the mobile phase induces the interference of the detection of sample component absorption bands, but some transparent region of the mid-IR range produces detection possibility. For example, if one uses a mobile phase of a deuterated solvent such as heavy water or perdeuterated methanol, IR can monitor many organic compounds that have C-H structures in the molecules.

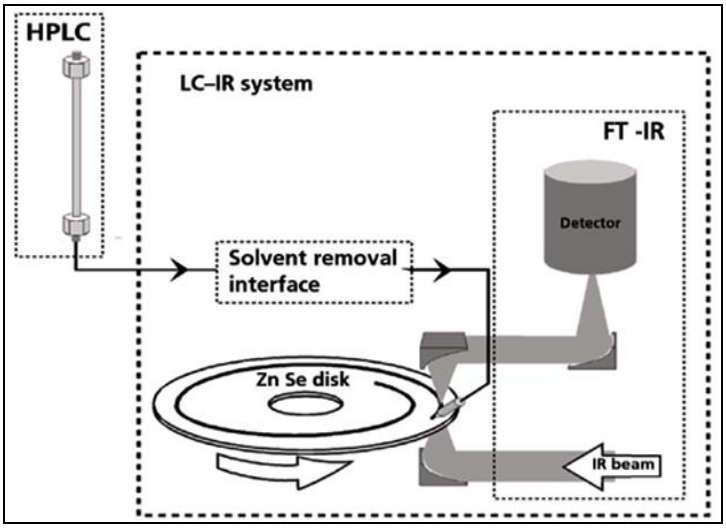

FIG. 3: LC-IR SYSTEM

The solvent-elimination approach is the preferred option in most of the LC-IR operations, the mobile phase solvent is eliminated, IR detection is carried out in some medium that has a transparency for IR light. Generally, KBr or KCl salts are used for the collection of sample components in the eluent, and heating up the medium before IR detection eliminates the volatile mobile phase solvents [5, 6]. There are two types of interfaces for the solvent-elimination approach:

- Diffuse-reflectance infrared Fourier transform (DRIFT) approach
- Buffer-memory technique.

Application:

A. Identification of organic compounds.

3. **LC-MS:** This hyphenated techniques LC-MS or HPLC-MS refers to the coupling of an LC (Liquid chromatography) with a mass spectrometer (MS)

Instrumentation: A switching valve can help make a working combination of the two techniques. A typical automated LC-MS system (Figure 4) consists of double three-way diverter in-line with an;

1. Auto sampler
2. LC system
3. The Mass spectrometer.

The diverter generally operates as an automatic switching valve to divert undesired portions of the eluate from the LC system to waste before the sample enters the MS. The ionization techniques used in LC-MS are generally soft ionization techniques that mainly display the molecular ion species with only a few fragment ions.

Hence, the information obtained from a single LC-MS run, on the structure of the compound, is rather poor. However, this problem has now been tackled by the introduction of tandem mass spectrometry (MS-MS), which provides fragments through collision-induced dissociation of the molecular ions produced. The use of LC-MS-MS is increasing rapidly.

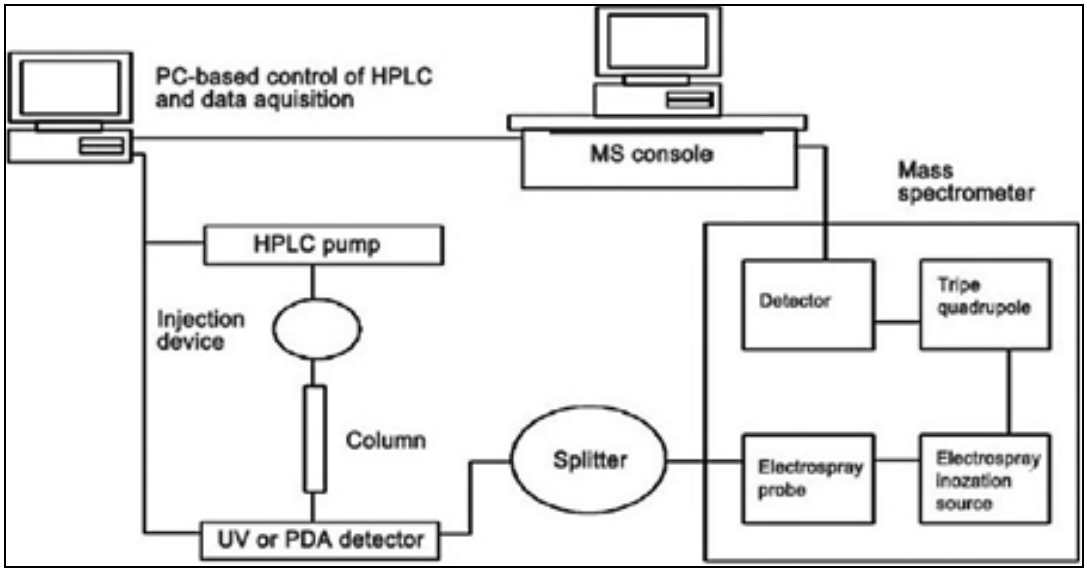

FIG. 4: TYPICAL LC-MS SYSTEM

An LC-MS combines the chemical separating power of LC with the ability of an MS to selectively detect and confirm molecular identity. MS is one of the most sensitive and highly selective methods of molecular analysis, and provides information on the molecular weight as well as the fragmentation pattern of the analyte molecule. The information obtained from MS is invaluable for confirming the identities of the analyte molecules [7, 8, 9].

Hyphenated techniques such as; HPLC coupled to UV and mass spectrometry (LC-UV-MS) have proved to be extremely useful in combination with biological screening for a rapid survey of natural products.

Application:

A. Selectively detect and confirm molecular identity.

B. Provides information on the molecular weight as well as the fragmentation pattern of the analyte molecule.

C. Help in identification of the analyte molecules.

D. This qualitative analysis makes it possible to reconstruct an unknown compound from MS data.

E. It is systematically applied to monitor impurity profiles during pharmaceutical development and scaling up and supports the safety evaluation of batches used in clinical studies.

4. **LC-NMR [2, 4]:** In this technique technological developments have allowed the direct parallel coupling of HPLC systems to NMR, giving rise to the new practical technique HPLC-NMR or LC-NMR, which has been widely known for more than last 15 years. The first on-line HPLC-NMR experiment using superconducting magnets was reported in the early 1980s. However, the use of this hyphenated technique in the analytical laboratories started in the latter part of the 1990s only.

   LC-NMR experiments can be performed in both continuous-flow and stop-flow modes. A wide range of bioanalytical problems can be addressed using 500, 600, and 800 MHz systems with 1H, 13C, 2H, 19F, and 31P probes. The main prerequisites for on-line LC-NMR, in addition to the NMR and HPLC instrumentation, are the continuous-flow probe and a valve installed before the probe for recording either continuous-flow or stopped-flow NMR spectra.

   A UV-Vis detector is also used as a primary detector for LC operation. Magnetic field strengths higher than 9.4 T are recommended, i.e., 1H resonance frequency of 400 MHz for a standard HPLC-NMR coupling. The analytical flow cell was initially constructed for continuous-flow NMR acquisition. However, the need for full structural assignment of unknown compounds, especially novel natural products, has led to the application in the stopped-flow mode.

Instrumentation: Generally, in LC-NMR system (**Figure 5**), the LC unit comprises

1. auto sampler,
2. LC pump,
3. column, and
4. Non-NMR detector (e.g., UV, DAD, EC, refractive index, or radioactivity).

From this detector, the flow is guided into the LC-NMR interface, which can be equipped with additional loops for the intermediate storage of selected LC peaks. The flow from the LC-NMR interface is then guided either to the flow-cell NMR probe-head or to the waste receptacle. Following passage through the probe-head, the flow is routed to a fraction collector for recovery and further investigation of the various fractions analyzed by NMR.

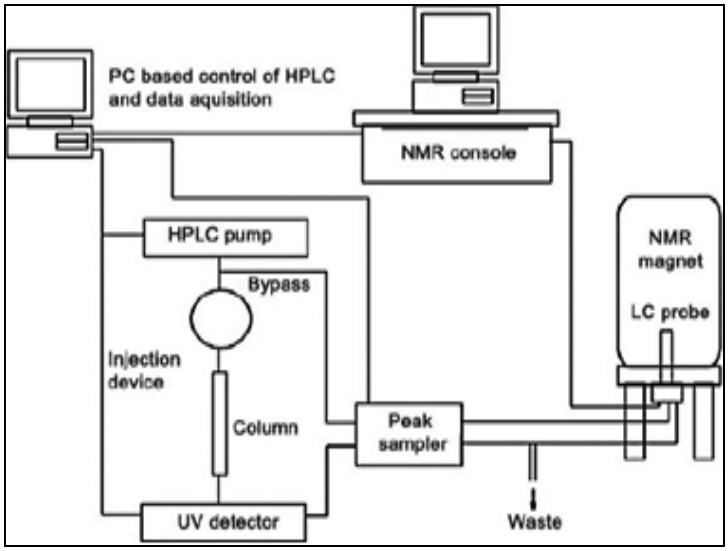

FIG. 5: LC- NMR SYSTEM

In most of the LC-NMR operations, reversed-phase columns are used, employing a binary or tertiary solvent mixture with isocratic or gradient elution. The protons of the solvents of the mobile phase cause severe problems for obtaining an adequate NMR spectrum. The receiver of the NMR spectrometer is not quite able to handle the intense solvent signals and the weak substance signals at the same time. To overcome this problem, solvent signal suppression can be achieved by one of the three major methods: presaturation, soft-pulse multiple irradiation or water suppression enhancement through T1 effects (WET) presaturation employing a z-gradient [10].

This problem can also be minimized by considering the following guidelines.

1. Using eluents that have as few 1H NMR resonances as possible, e.g., H_2O, ACN, or MeOH.
2. Using at least one deuterated solvent, e.g., D2O (approx $290/L), ACN-d3 (approx. $1600/L), or MeOD (approx. $3000/L).
3. Using buffers that have as few 1H NMR resonances as possible, e.g., TFA or ammonium acetate.
4. Using ionpair reagents that have as few 1H NMR resonances as possible, e.g., ionpairs with *t*-butyl groups create an additional resonance.

Application:

A. It provides information toward the structure elucidation of natural products.

B. The analysis of complex mixtures of all types, particularly the analysis of natural products and drug-related metabolites in biofluids.

5. **CE-MS:** When an MS detector is linked to a CE system for acquiring on-line MS data of the separated compound, the resulting combination is termed as CE-MS (**Figure 6**). CE is an automated separation technique introduced in the early 1990s. CE analysis is driven by an electric field, performed in narrow tubes, and can result in the rapid separation of many hundreds of different compounds. The versatility and the many ways that CE can be used mean that almost all molecules can be separated using this powerful method.

   It separates species by applying voltage across buffer-filled capillaries, and is generally used for separating ions that move at different speeds when voltage is applied, depending on their size and charge. The solutes are seen as peaks as they pass through the detector and the area of each peak is proportional to their concentration, which allows quantitative determinations. Analysis includes purity determination, assays, and trace level determinations [11].

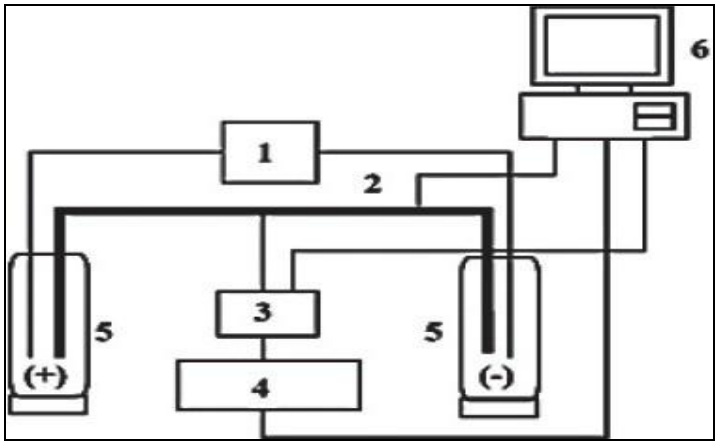

FIG. 6: A TYPICAL CE-MS SYSTEM

Instrumentation: It consists of;

1. High-Voltage Supply;
2. Capillary
3. UV-Vis or PDA detector
4. MS detector;
5. Buffer solution
6. PC control

Application:

A. Rapid separation of many hundreds of different compounds.

Application of Hyphenated Technique in Pharmacy [12, 13, 14, 15, 16]**:** Some examples of the application of hyphenated techniques in natural products analysis are (**Figure 7**).

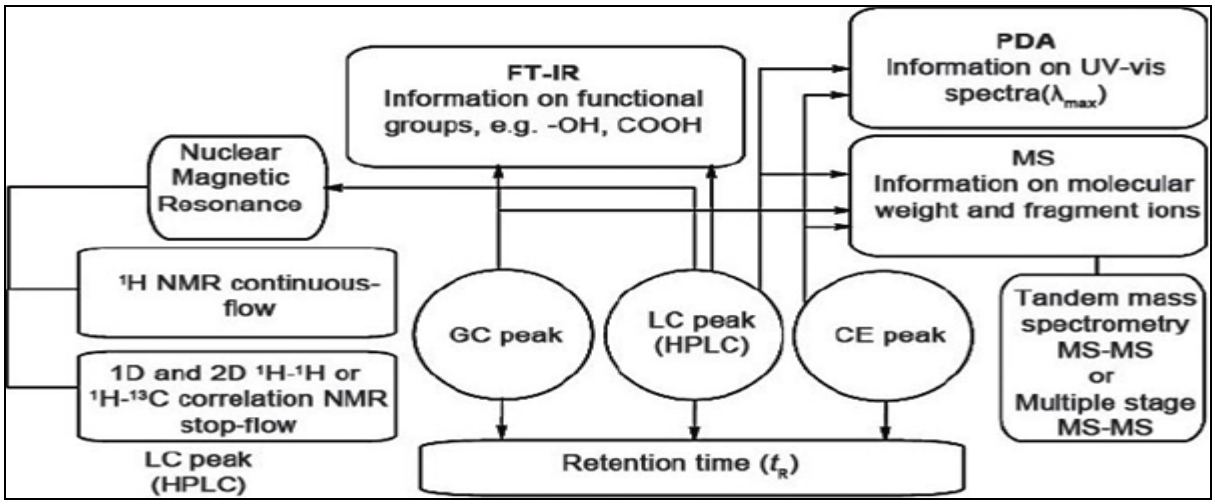

FIG. 7: REVIEW OF APPLICATION OF HYPHENATED TECHNIQUE

1. **Isolation and Analysis of Natural Products:** Crude natural product extracts, which represent extremely complex mixtures of numerous compounds, can be analyzed successfully by using appropriate hyphenated techniques. Among the various hyphenated techniques, LC-MS are the two most extensively used for natural product analysis. LC-NMR, as well as different multiple hyphenated techniques like LC-NMR-MS have also become popular most recently.

   LC-MS, if the ionization technique is chosen appropriately, can be an extremely powerful and informative tool for screening crude plant extracts. The currently available various types of LC-MS systems allow the analysis of small nonpolar compounds to large polar constituents like oligosaccharides, proteins, and tannins present in natural product extracts.

- Alkaloids are a large group of nitrogen-containing secondary metabolites of plant, microbial, or animal origin. Various hyphenated techniques have been used in the analysis of several types of alkaloids to date. GC-MS has become the method of choice for the analysis of various pyrolizidine and quinolizidine types of alkaloids.

- The coumarins are the largest class of 1-benzopyran derivatives that are found mainly in higher plants. HPLC-PDA can be used successfully in the analysis of various phenolic compounds, including coumarins, because of the presence of significant amounts of chromophores in these molecules. The HPLC-PDA determination of coumarins, where absorption spectra are registered with a PDA detector, provides useful information about the identity of the molecule including oxidation pattern.

- GC-MS has been demonstrated to be a valuable analytical tool for the analysis of mainly nonpolar components and volatile natural products, e.g., mono- and sesquiterpenes. Chen *et al*. described a method using direct vaporization GC-MS to determine approx 130 volatile constituents in several Chinese medicinal herbs. They reported an efficient GC-MS method for the separation and structure determination of the constituents in ether-extracted volatile oils of Chinese crude drugs, Jilin Ginseng, Radix aucklandiae, and Citrus tangerina peels.

- Saponins are steroidal or triterpenoidal glycosides that occur widely in plant species of nearly 100 families. As saponins are highly polar compounds and difficult to volatilize, the application of GC-MS is mainly restricted to the analysis of aglycones known as sapogenins or saponins. Sometimes, precolumn derivatization of saponins can be used to attach a chromophore that facilitates UV detection at higher wavelengths. Among the hyphenated techniques, LC-MS, LC-NMR, and CE-MS could be useful for the rapid initial screening of crude extracts.

2. **Chemical Fingerprinting and Quality Control of Herbal Medicine** [17, 18]: The use of hyphenated techniques, e.g., LC-MS, CE-MS, LC-NMR, or LC-NMR-MS, in chemical fingerprinting analysis for quality control and standardization of medicinal herbs has attracted immense interest in recent years. Generally, in the context of drug analysis, fingerprinting method is used to highlight the profiles of the sample matrix, which is often sufficient to provide indications of the source and method of preparation. In herbal medicines, the profile depends not only on the preparation processes but also on the quality of the crude herb source material.

3. **Analytical Chemistry:** It is useful in determination of drug and identification of its degraded products. It is systematically applied to monitor impurity profiles during pharmaceutical development and scaling up and supports the safety evaluation of batches used in clinical studies.

4. **Chemotaxonomy:** Chemical taxonomy or chemotaxonomy is based on the principle that the presence of certain secondary metabolites is dictated by various enzymes involved in the biosynthesis of these compounds. Hence, chemical profiling of these secondary metabolites, either by complete isolation and identification, or by separation and on-line identification using modern hyphenated techniques, could provide useful information with regard to the taxonomic or even phylogenetic relationships among various species.

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