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### ANALYTICAL TECHNIQUES (CHROMATOGRAPHY, SPECTROSCOPY, ELECTROPHORESIS) IN PHARMACEUTICAL ANALYSIS: A REVIEW

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#### ABSTRACT

The development of the pharmaceuticals brought a revolution in human health. These pharmaceuticals would serve their intent only if they are free from impurities and are administered in an appropriate amount. To make drugs serve their purpose various chemical and instrumental methods were developed at regular intervals which are involved in the estimation of drugs. These pharmaceuticals may develop impurities at various stages of their development, transportation and storage which makes the pharmaceutical risky to be administered, thus they must be detected and quantitated. For this analytical instrumentation and methods play an important role. This review highlights the role of the analytical instrumentation and the analytical methods in assessing the quality of the drugs. The review highlights analytical techniques such as chromatography, spectroscopy, electrophoresis and their corresponding methods that have been applied in the analysis of pharmaceuticals.

#### KEY WORDS

Analytical Instruments, Pharmaceuticals, Quality, Chromatography, Spectroscopy and Electrophoresis.

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#### INTRODUCTION

The process of drug development starts with the innovation of a drug molecule that has showed therapeutic value to battle, control, check or cure diseases. The synthesis and characterization of such molecules which are also called active pharmaceutical ingredients (APIs) and their analysis to create preliminary safety and therapeutic efficacy data are prerequisites to identify the drug candidates for further detailed investigations.

In the field of pharmaceutical research, the analytical investigation of bulk drug materials, intermediates, drug products, drug formulations, impurities, degradation products, and biological samples containing the drugs and their metabolites is very important. From the commencement of official pharmaceutical analysis, analytical assay methods were included in the compendial monographs with the aim to characterize the quality of bulk drug materials by setting limits of their active ingredient content. In recent years, the assay methods in the monographs include titrimetry, spectrometry, chromatography, and capillary electrophoresis; also the electro analytical methods can be seen in the literature.

From the stages of drug development to marketing and post marketing, analytical techniques play a great role, be it understanding the physical and chemical stability of the drug, impact on the selection and design of the dosage form, assessing the stability of the drug molecules, quantitation of the impurities and identification of those impurities which are above the established threshold essential to evaluate the toxicity profiles of these impurities to distinguish these from that of the API, when applicable and assessing the content of drug in the marketed products. The analysis of drug and its metabolite which may be either quantitative or qualitative is extensively applied in the pharmacokinetic studies.

This review highlights the role of various analytical techniques and their corresponding analytical methods in the analysis of pharmaceuticals.

## **ANALYTICAL TECHNIQUES**

### **Chromatographic Techniques**

Chromatography is an analytical technique based on the separation of molecules due to differences in their structure and/or composition. In general, chromatography involves moving a sample through the system over a stationary phase. The molecules in the sample will have different affinities and interactions with the stationary support, leading to separation of molecules. Sample components that display stronger interactions with the stationary

phase will move more slowly through the column than components with weaker interactions. Different compounds can be separated from each other as they move through the column<sup>1</sup>.

### **THIN LAYER CHROMATOGRAPHY**

In thin layer chromatography, a solid phase (stationary phase), the adsorbent, is coated onto a solid support as a thin layer (about 0.25 mm thick). In many cases, a small amount of a binder such as plaster of Paris is mixed with the adsorbent to facilitate the coating. Many different solid supports are employed, including thin sheets of glass, plastic, and aluminum. The mixture (A plus B) to be separated is dissolved in a solvent and the resulting solution is spotted onto the thin layer plate near the bottom. A solvent, or mixture of solvents, called the eluents, is allowed to flow up the plate by capillary action. At all times, the solid will adsorb a certain fraction of each component of the mixture and the remainder will be in solution. Any one molecule will spend part of the time sitting still on the adsorbent with the remainder moving up the plate with the solvent. A substance that is strongly adsorbed (say, A) will have a greater fraction of its molecules adsorbed at any one time, and thus any one molecule of A will spend more time sitting still and less time moving. In contrast, a weakly adsorbed substance (B) will have a smaller fraction of its molecules adsorbed at any one time, and hence any one molecule of B will spend less time sitting and more time moving. Thus, the more weakly a substance is adsorbed, the farther up the plate it will move. The more strongly a substance is adsorbed, the closer it will stay near the origin.

### **HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)**

#### **HPLC: A Powerful Separation Method**

A powerful separation method must be able to resolve mixtures with a large number of similar analytes. A chromatogram provides directly both qualitative and quantitative information: each compound in the mixture has its own elution time (the point at which the signal appears on the screen) under a given set of conditions; and both the area

and height of each signal are proportional to the amount of the corresponding substance. This example shows that high-performance liquid chromatography (HPLC) is very efficient, i.e. it yields excellent separations in a short time. The 'inventors' of modern chromatography, Martin and Syngé were aware as far back as 1941 that, in theory, the stationary phase requires very small particles and hence a high pressure is essential for forcing the mobile phase through the column. As a result, HPLC was sometimes referred to as high-pressure liquid chromatography<sup>3</sup>.

## **SPECTROSCOPIC TECHNIQUES**

### **Spectrophotometry**

Another important group of methods which find an important place in pharmacopoeias are spectrophotometric methods based on natural UV absorption and chemical reactions. Spectrophotometry is the quantitative measurement of the reflection or transmission properties of a material as a function of wavelength.

The advantages of these methods are low time and labor consumption. The precision of these methods is also excellent. The use of UV-Vis spectrophotometry especially applied in the analysis of pharmaceutical dosage form has increased rapidly over the last few years<sup>4</sup>.

The Light of Knowledge is an often used phrase, but it is particularly appropriate in reference to spectroscopy. Most of what we know about the structure of atoms and molecules comes from studying their interaction with light (electromagnetic radiation). Different regions of the electromagnetic spectrum provide different kinds of information as a result of such interactions.

### **Mass Spectrometry**

Sample molecules are ionized by high energy electrons. The mass to charge ratio of these ions is measured very accurately by electrostatic acceleration and magnetic field fluctuations, providing a precise molecular weight. Ion fragmentation patterns may be related to the structure of the molecular ion.

### **Ultraviolet-Visible Spectroscopy**

Absorption of this relatively high-energy light causes excitation of electrons. The easily accessible part of this region (wavelengths of 200 to 800 nm) shows absorption only if conjugated pi-electron systems are present.

### **Infrared Spectroscopy**

Absorption of this lower energy radiation causes vibrational and rotational excitation of groups of atoms within the molecule. Because of their characteristic absorptions identification of functional groups is easily accomplished.

### **Nuclear Magnetic Resonance Spectroscopy**

Absorption in the low-energy radio-frequency part of the spectrum causes excitation of nuclear spin states. NMR spectrometers are tuned to certain nuclei (e.g. <sup>1</sup>H, <sup>13</sup>C, <sup>19</sup>F and <sup>31</sup>P). For a given type of nucleus, high-resolution spectroscopy distinguishes and counts atoms in different locations in the molecule<sup>5</sup>.

### **HPLC Coupling With Various Spectroscopic Techniques**

HPLC can be combined with numerous other analytical techniques but the most important coupling principle is the one with spectroscopy. Chromatography and spectroscopy are orthogonal techniques, i.e. their types of information are very different. Chromatography is a separation method and spectroscopy is a technique which yields a 'fingerprint' of molecules. Coupling with atomic spectrometry is rarely used although it allows the detection of toxic metals in environmental samples or of metalloproteins<sup>6</sup>. Four other techniques, HPLC-UV, HPLC-FTIR, HPLC-MS and HPLC-NMR are more important because excellent spectra are obtained with them, thus allowing structure elucidation.

### **HPLC-UV: The Diode Array Detector<sup>7</sup>**

Compared to conventional UV detectors, the diode array detector is built with inverse optics. Figure No.1 shows the principle of this instrument. The full light first goes through the detector cell and is subsequently divided spectrally in a polychromator (which is a grating). The spectral light then reaches the diode array, a chip with a large.

Number (100-1000) of light-sensitive diodes that are arranged side by side. Each diode only obtains a well defined fraction of the information which is read by the electronics for data processing. This type of detector allows a wealth of information to be obtained from UV spectra. The most important possibilities are presented in Figure No.2:

- It is possible to obtain and store spectra of individual peaks during a chromatogram. This process does not take more than half a second, including data processing; therefore it is possible to obtain several spectra, even from narrow peaks. For identification the spectra can be compared on-line with a library. In order to improve correlations, the computer calculates the second derivative which shows more maxima and minima than the original spectrum.
- The knowledge of spectra of the compounds involved then allows detection at selected wavelengths. Interfering peaks can be eliminated due to the proper choice of detection conditions. It is often possible to obtain an accurate quantitation of a compound even if the resolution in principle is poor. The detection wavelength can be altered during a chromatographic run<sup>4</sup>.
- Simultaneous detection at two different wavelengths allows calculation of the absorbance ratio<sup>8</sup>. If this ratio is constant over the whole width of a peak it can be assumed that it is pure (with the exception of the exact coincidence of two peaks with identical shape or of an impurity with identical UV spectrum). If the ratio is not constant this is proof that the peak is not pure<sup>4</sup>.
- Subtraction of two wavelengths allows baseline drift to be reduced during gradient elution as well as noise. The reference wavelength can be chosen in a region where none of the compounds of interest shows absorption.

#### HPLC-FTIR<sup>9</sup>

The coupling with Fourier-transform infrared spectroscopy allows spectra to be obtained. The detection limit is rather high (i.e. poor) if a flow-through cell is used. The interfaces with solvent

elimination are more interesting and flexible but technically more demanding; they can even register spectra of trace analytes. A volatile mobile phase is needed; it may be aqueous.

#### HPLC-MS<sup>10</sup>

Mass spectrometers for HPLC consist of three different parts: the interface where the eluate enters the MS and the ions are generated, the mass analyzer, and the detector, an electron multiplier which determines the ion beam intensity. Thanks to the different possibilities for ion generation at atmospheric pressure, HPLC-MS is possible with rather low expenditure.

The main two ionization techniques are APCI and ESI.

**APCI: Atmospheric pressure chemical ionization** (Figure No.3).

- Ions are generated by corona discharge (3-6 kV).
- Yields molecule ions  $(M + H)^+$  (no spectra), negative ionization is also possible.
- Suitable for small, medium and nonpolar molecules; however, they need some proton affinity and volatility.
- Not suitable for thermolabile analytes,
- For aqueous and nonaqueous mobile phases, flow must be at least  $1 \text{ ml min}^{-1}$ . With nonaqueous eluents no additives are necessary; reactions with the solvent are possible during ionization.
- With aqueous eluents an additive may be necessary for efficient ionization.
- Mass-sensitive signal.

**ESI or APESI: (Atmospheric pressure) electrospray ionization** (Figure No.4).

- Ions are generated by 'coulomb explosion' (disintegration) of electrically charged droplets.
- Yields ions with single or multiple charge; in the latter case, spectra with many peaks are obtained which must not be mixed up with classical spectra showing molecule fragments.
- Suitable for thermolabile analytes and macromolecules, including biopolymers.
- Suitable for aqueous eluents; flow must be small, therefore useful for micro HPLC.

- For positive ionization a pH of 5 is suitable, additives are formic and acetic acid, perhaps together with ammonium acetate:



- For negative ionization a pH of 9 is suitable, additives are ammonia, triethylamine and diethylamine, perhaps together with ammonium acetate:



Concentration-sensitive signal.

Quadrupoles and ion traps are the most frequently used mass analyzers. Both systems are rather low-priced and robust but their resolution is limited to 1Da. The ions can be sent through several mass analysers and fragmentations, setup in series, thus yielding more information about the structure. These techniques are known as MS/MS (two steps) or MSn (n steps).

#### HPLC-NMR<sup>11</sup>

Nuclear magnetic resonance can be coupled with HPLC if the solvents are chosen properly (one solvent signal can be suppressed but the others must be avoided by using deuterated solvents) and if the analyte concentration is not too low. Detection limit depends on radiofrequency; therefore a 500-800MHz instrument must be used. If the analyte concentration is high enough, it is possible to run online HPLC-NMR separations, but usually the individual peaks are stored in loops and spectra are obtained offline with a long enough measuring time<sup>4</sup>.

#### Comparison between High-Performance Liquid Chromatography and Gas Chromatography

Like HPLC, gas chromatography<sup>12</sup>(GC) is also a high-performance method, the most important difference between the two being that GC can only cope with substances that are volatile or can be evaporated intact at elevated temperatures or from which volatile derivatives can be reliably obtained. Only about 20% of known organic compounds can be analysed by gas chromatography without prior treatment. For liquid chromatography, the sample must be dissolved in a solvent and, apart from cross-linked, high-molecular-mass substances; all organic and ionic inorganic products satisfy this condition.

The characteristics of the two methods are compared in Table No.2. In comparison with gas chromatography there are three important differences:

- The diffusion coefficient of the sample in the mobile phase is much smaller in HPLC than in GC. (This is a drawback because the diffusion coefficient is the most important factor which determines the speed of chromatographic analysis).
- The viscosity of the mobile phase is higher in HPLC than in GC. (This is a drawback because high viscosity results in small diffusion coefficients and in high flow resistance of the mobile phase).
- The compressibility of the mobile phase under pressure is negligibly small in HPLC whereas it is not in GC. (This is an advantage because as a result the flow velocity of the mobile phase is constant over the whole length of the column. Therefore optimum chromatographic conditions exist everywhere if the flow velocity is chosen correctly. Moreover, incompressibility means that a liquid under high pressure is not dangerous).

#### Comparison between High-Performance Liquid Chromatography and Capillary Electrophoresis

Capillary electrophoresis<sup>13</sup> (also termed capillary zone electrophoresis, CZE) is suited for electrically charged analytes and separates them, simply speaking, according to their ratio of charge to size. In addition, the shape of the molecules is another parameter which influences their speed; therefore the separation of isomers or of analytes with identical specific charge is possible. Cations (positively charged molecules) move faster than anions (negatively charged molecules) and appear earlier in the detector. Small, multiply charged cations are the fastest species whereas small, multiply charged anions are the slowest ones.

The separation is performed at high voltage. An electric field of up to 30 kV is applied between the ends of the separation capillary. As a consequence, the buffer solution within the capillary moves towards the negatively charged cathode. The

capillaries have a length of 20-100 cm and an inner diameter of 50-250  $\mu$ m. In contrast to HPLC they are not packed with a stationary phase in the chromatographic sense but in some cases with a gel which allows the separation of the analytes by their size (as in size-exclusion chromatography).

The separation performance can be of much higher order of magnitude than in HPLC (up to  $10^7$  theoretical plates), making CE an extremely valuable method for peptide mapping or DNA sequencing. However, small molecules such as amino acids or inorganic ions can be separated as

well. The absolute sample amounts which can be injected are low due to the small volume of the capillaries. A major drawback is the lower repeatability (precision) compared to quantitative HPLC. Preparative separations are not possible.

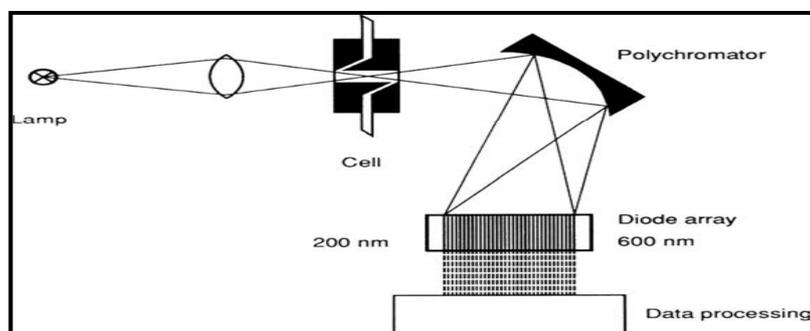
Electrokinetic chromatography is a hybrid of HPLC and CE. For this technique the capillaries are packed with a stationary phase and the separation is based on partition phenomena. The mobile phase acts as in CE; it consists of a buffer solution and moves thanks to the applied electrical field<sup>4</sup>.

**Table No.1: Lists a number of adsorbents in the order of adsorptive power<sup>2</sup>**

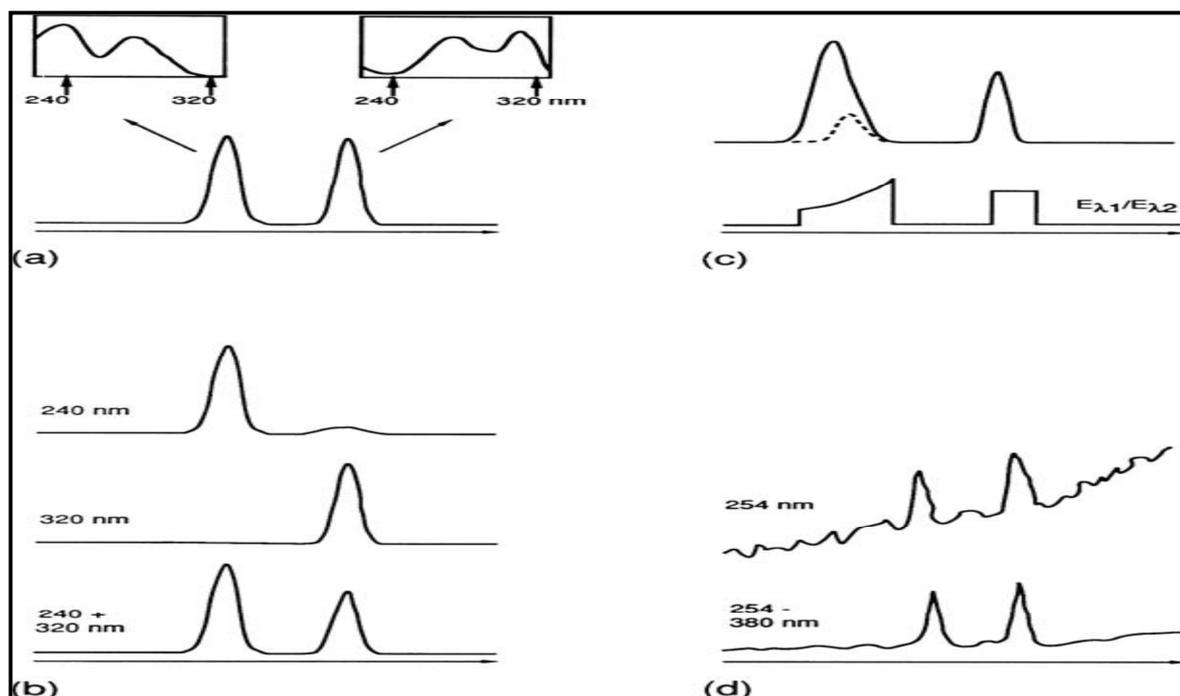
Most Strong Adsorbent	Alumina Charcoal Florisil	Al <sub>2</sub> O <sub>3</sub> C MgO/SiO <sub>2</sub> (Anhydrous)
Least Strong Adsorbent	Silica Gel	SiO <sub>2</sub>

**Table No.2: Comparison of GC and HPLC<sup>4</sup>**

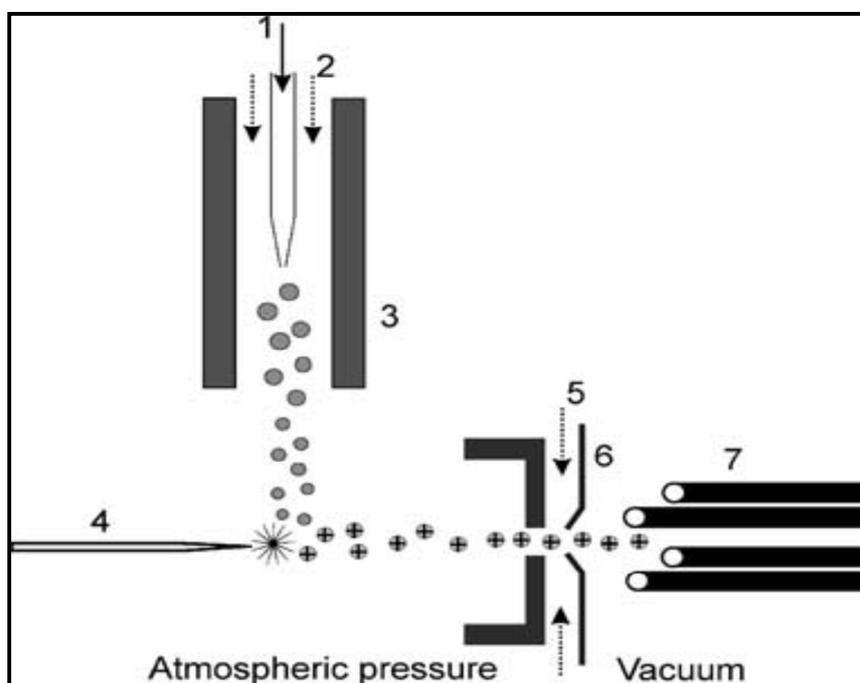
S.No	Problem	GC	HPLC
1	Difficult separation	Possible	Possible
2	Speed	Yes	Yes
3	Automation	Possible	Possible
4	Adaptation of system to separation problem	By change in stationary phase	By change in stationary and mobile phase
5	Application restricted by	Lack of volatility, thermal decomposition	Insolubility
6	Typical number of separation plates	Per column	Per metre
7	GC with packed columns	2000	1000
8	GC with capillary columns	50 000	3000
9	Classical liquid Chromatography	100	200
10	HPLC	5000	50 000



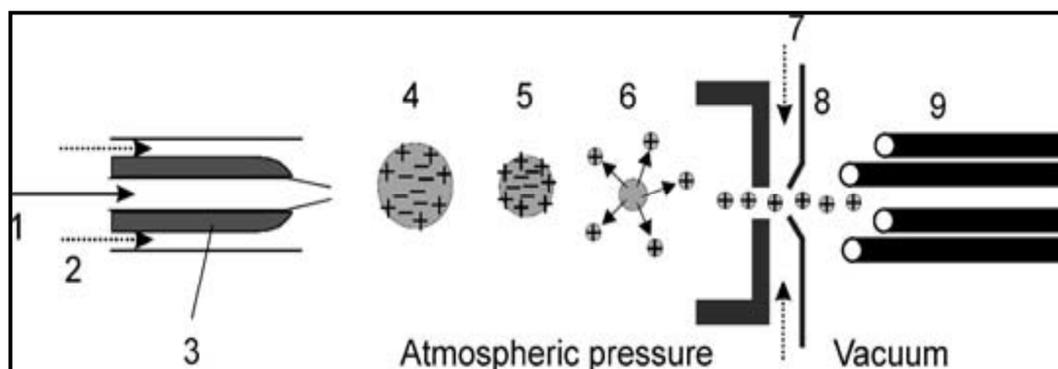
**Figure No.1: Principle of a diode array detector<sup>4</sup>**



**Figure No.2: Possibilities of the diode array detector. (a) Registration of spectra during the separation; (b) registration of a chromatogram at different wavelengths; (c) peak purity control; (d) chromatogram with and without subtraction of a reference wavelength<sup>4</sup>**



**Figure No.3: HPLC-MS interface for APCI. 1¼ from HPLC column; 2¼ nebulizing gas; 3¼ heater; 4¼ corona discharge needle; 5¼ drying gas; 6¼ skimmer; 7¼ quadrupole.<sup>4</sup>**



**Figure No.4: HPLC-MS interface for ESI. 1¼from HPLC column; 2¼ nebulizing gas; 3¼ high voltage; 4¼ charged droplet (drawn too large); 5¼evaporation; 6¼ coulomb explosion; 7¼ drying gas; 8¼ skimmer; 9¼quadrupole.<sup>4</sup>**

## CONCLUSION

Chromatography and spectroscopy are orthogonal techniques, i.e. their types of information are very different. Chromatography is a separation method and spectroscopy is a technique which yields a 'fingerprint' of molecules. HPLC is a technique for separation, identification and quantification of components in a mixture. It is especially suitable for compounds which are not easily volatilized, thermally unstable and have high molecular weights. But whereas GC is effectively used for analysis of gas mixtures or liquids having low boiling points. On the other hand liquid chromatography is applied for separation of thermally labile liquid mixtures or those having high boiling points.

The advantage of UV method over HPLC method is that the UV method does not require the elaborate treatment and procedures usually associated with chromatographic method. It is less time consuming and economical. The HPLC and UV spectrometry methods are adequate methods to quantify a drug in pure form and its dosage form. Since these methods are simple, specific, rapid, precise and accurate, they may be successfully and conveniently adopted for routine quality control analysis of drugs in bulk and pharmaceutical dosage form.

A major drawback of CE is the lower repeatability (precision) compared to quantitative HPLC.

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## CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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