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CAPILLARY ELECTROPHORESIS INDUCTIVELY COUPLED MASS SPECTROMETRY

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ABSTRACT

Capillary electrophoresis (CE) is a separation technique in which the analytes are separated on the basis of differences in their charge-to-size ratios. This makes CE especially suitable for the analysis of molecules with abroad range of sizes, charge and hydrophobicity, as proteins and peptides. Another attractive feature of CE is its ability to handle minute sample amounts, with nano-litre injection volumes, which makes it ideal for applications when the sample volumes are limited, as often is the case in analysis of body fluids, single cells and other small volume analysis of biofluids. Mass spectrometry (MS), is today one of the most advanced detection techniques. It yields maximum accurate information in short time, consuming minimal amounts of sample. MS provides robustness and reproducibility of analysis as well as high throughput possibilities via automatic data acquisition. When combined with ionization techniques like inductively coupled plasma (ICP), electro spray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), MS allows simultaneous analysis of proteins and peptides with high resolution. However, in order to reach ultimate mass sensitivity a separation step prior to MS detection is often needed. The possibility of combining the properties of MS with a fast and high-efficiency separation provided by CE to meet the analytical challenges, that the complexity of biological samples represents, has been recognized for about two decades.

Keywords: Capillary electrophoresis, ICP, Coupling.

INTRODUCTION

Electrophoresis is a separation method based on the differential rate of migration of charged species in an applied DC electric field. Electrophoresis on a marco scale has been applied to a variety of difficult analytical separation problems: proteins, peptides, nucleotides, polynucleotide's, amino acids, cations and anions. Electrophoresis has unique ability to separate charged macro molecules of interest in biochemical, biological, biomedical research and the biotechnology industry. For many years, electrophoresis has been the power house method for separating proteins (enzymes, hormones, nucleic acids). For example to sequence DNA it is necessary to distinguish between long-chain polynucleotide's that have as many as perhaps 200 to 500 bases and that differ by only a single nucleotide. Only electrophoresis has sufficient resolving power to handle this problem [1].Electrophoresis separations are currently performed in two different formats: one is called *slab electrophoresis* and the other *capillary electrophoresis.*

Basis of electrophoresis

The migration rate of *v* of an ion(cm/s) in an electric field is equal to the product of the field strength E and the electrophoretic mobility μe . That is

$$v = \mu e\, E$$

The electrophoretic mobility is In turn proportional to the ionic charge on the analyte and inversely proportional to frictional retarding factors. The electric field acts on only ions. If two species differ either in charge or in the frictional forces they experience while moving through the buffer, they will be separated from each other. Neutral species are not separated. The frictional retarding force on an analyte ion is determined by the size and shape of the ion and the viscocity of the migration medium. For ions of

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the same *size*,the greater the charge, the greater the driving force and the faster the rate of migration. For ions of the same charge, the smaller the ion, the smaller the frictional forces and the faster the rate of migration. The ion's *charge-to-size* ratio combines these two effects [1].

Capillary Electrophoresis

Basic theory of CE

Separation by electrophoresis is based on differential movement of charged species (ions) by attraction or repulsion in an electric field. In CE, electrophoretic separation is performed in capillaries made of fused silica (a metal free glass material), typically 25-75 μm in inner diameter, which are usually filled with a buffer. The high electrical resistance of the capillary enables the application of very high electrical fields (100-500 V/cm) with only minimal heat generation. The use of high electrical fields results in short analysis times and high peak efficiency and resolution. Other properties that characterise CE are the minimal sample volume requirements (low nanolitre range) and its diversity of application.

Migration rates in CE

The migration rate of an ion *v* depends on the electric field strength. The electric field in turn is proportional to the magnitude of the applied voltage *V* and inversely proportional to the length *L* over which it is applied. Thus

$v = \mu e\ V/L$

The relationship indicates that high applied voltages are desirable to achieve rapid separation .[1]

Plate heights in CE

Although CE is not a chromatographic process, separations are often described in a manner similar to chromatography. For example, in electrophoresis, we calculate the plate count *N* by

$N = \mu e\ V/2D$,

Where D is the diffusion coefficient of the solute .Because resolution increases as the plate count increases, it is desirable to use high applied voltage to achieve high-resolution separations. Note that for electrophoresis, contrary to the situation in chromatography, the plate count does not increase with the length of the column. High-voltage power supplies of 10-25kV are normal. CE normally yields plate counts in the range of 1, 00,000 to 2, 00,000 .[1]

CE instrumentation

One of the key features of CE is the simplicity of the instrumentation. A CE system consists of a buffer-filled fused silica capillary, where the separation takes place, two buffer reservoirs connected to a high voltage power supply using platinum electrodes and a detector, usually placed closer to the outlet buffer reservoir, connected to a computer for data collection. A basicInstrumental set-up of CE is shown in Figure 1.

Sample is loaded into the capillary on the opposite side of the detector. Upon application of a potential difference over the capillary (usually 15-30 kV) the separation is started. Ultraviolet (UV) detectors are most commonly used, but numerous other detection techniques can be employed. Mass spectrometry is, however, the most universal and presently unrivalled detection technique that is the best choice in combination with separation in the same system. In this thesis CE is interfaced to MS using ICP ionization.

Electrically driven separation in CE (EOF)

The mechanisms responsible for separation in CE are different from those in chromatography. In CE there is a specific electrophoretic phenomenon called electro osmotic flow (EOF) which simply can be considered as an electric pump that continuously pushes the solvent inside the capillary towards the detector. This happens when the inner wall surface of the silica capillary, consisting of protolyticsilanol groups (Si-OH, pKa ~3-9), becomes activated. The activation of the wall starts with the deprotonation of silanol groups (to anionic form, Si-O-) when solvents with pH > 3 are used, giving a negatively charged surface. The hydrated cations from the solvent start to accumulate near the surface to maintain charge balance forming an electric *double layer* at the inner wall of the capillary.When the voltage is applied across the capillary the cations are attracted towards the cathode and start to move in that direction. Because they are solvated their movement drags the bulk solution in the capillary towards the cathode, giving rise to formation of the EOF. The EOF causes movement of nearly all species, regardless of charge, in the same direction. Thus, cations, neutrals and anions can be separated in a single run if the magnitude of the EOF is greater than the electrophoretic mobility's of anions in opposite direction. This process is depicted in Figure 3. Besides the different types of charges, other properties of analytes like their size and their net charge give rise to the differences in their migration times at a given pH of the BGE (background electrolyte) and the voltage applied over the capillary. In this way analytes achieve different velocities through the capillary and separate from each other.Bulk flow towards the cathode upon application of an electric field. Development of the electroosmotic flow.

Inductively coupled plasma mass spectrometry

Mass spectrometry has today a central role in life science, as it has become the analytical technique of choice in many application areas. MS methods often provide high sensitivities of analysis and produces data that is characteristic for the structure of the analytes. The analysis of a sample with MS requires that the molecules are electrically charged (ionized) and in the gas phase. Heating

of a sample to get it into the gas phase can easily be accomplished. However, analytes such as peptides and proteins are decomposed by heating. Therefore a ionization/desorption technique is required to produce intact molecular ions in gas phase. Today, there are a wide variety of MS-based strategies suitable for the analysis of different types of samples. In protein and peptide analysis, the most commonly used technique is ICP. Although mass spectrometry has a good capacity to analyse complex biological samples, great improvements in sensitivity and selectivity can be achieved by implementing a separation step prior to MS analysis. Furthermore, when ions are formed in the ion source there is always a competition for charge among the analyte molecules. Analytes with low affinity for charge and/or low abundance will be outclassed in the ionization process, which results in signal suppression and a biased analysis. A separation step reduces the number of components that are introduced into the ion source simultaneously, thereby reducing signal suppression. Capillary electrophoresis is a high resolving separation technique suitable for the analysis of small sample volumes and a wide variety of analytes. This technique enables separation, in electrophoretic mobility and size-to-charge ratio, in the same analytical system, providing a great
potential for analysis of complex biological sample.

Sample Ionization Systems

Inductively Coupled Plasma

Inductively coupled plasma (ICP) for spectrometry is sustained in a torch that consists of three concentric tubes, usually made of quartz. The end of this torch is placed inside an induction coil supplied with a radio-frequency electric current. A flow of argon gas (14 to 18 liters per minute) is introduced between the two outermost tubes of the torch and an electric spark is applied for a short time to introduce free electrons into the gas stream. These electrons interact with the radio-frequency magnetic field of the induction coil and are accelerated first in one direction, then the other, as the field changes at high frequency (usually 27.12 million cycles per second). The accelerated electrons collide with argon atoms, and sometimes a collision causes an argon atom to part with one of its electrons. The released electron is in turn accelerated by the rapidly changing magnetic field. The process continues until the rate of release of new electrons in collisions is balanced but the rate of recombination of electrons with argon ions (atoms that have lost an electron). This produces a 'fireball' that consists mostly of argon atoms with a rather small fraction of free electrons and argon ions. The temperature of the plasma is very high, of the order of 10,000 K. The ICP can be retained in the quartz torch because the flow of gas between the two outermost tubes keeps the plasma away from the walls of the torch. A second flow of argon (around 1 liter per minute) is usually introduced between the central tube and the intermediate tube to keep the plasma, away from the end of the central tube. A third flow (again usually around 1 liter per minute) of gas is introduced into the central tube of the torch. This gas flow passes through the centre of the plasma. Where it forms a channel that is cooler than the surrounding plasma but still much hotter than a chemical flame. Samples to be analyzed are introduced into this central channel, usually as a mist of liquid formed by passing the liquid sample into a nebulizer. As a droplet of nebulizer sample enters the central channel of the ICP , it evaporates and any solids that were dissolved in the liquid vaporize and then breakdown into atoms. At the temperatures prevailing in the plasma a significant proportion of the atoms of many chemical elements are ionized, each atom losing its most loosely bound electron to form a singly charged ion [17].

Electrostatic Acclerating System & Magnetic Field

Positively charged ions produced in the ionization chamber are acted upon by the electric field and forced to pass through the slit of the first accelerator plate owing to the potential difference between them. These ions attain their final velocities after passing through the second acceleratorplate. Potential difference of about 400-4000 V between the first and second accelerating plates enhances the velocities of ions.These accelerated ions are then passed through a curvature under the influence of magnetic field (H). Radius of the curvature (r) depends upon the mass of the ion (m),accelerating voltage(v),charge of the electron (e) and strength of the magnetic field(H). Mass to charge ratio (m/e) and radius of the curvature are the parameter upon which mass spectrometry is interdependent.

Mass Analyzers

Quadrupole

This analyzer is more compact and less expensive, more rugged. The heart of a quadrupole instrument is the four parallel cylindrical .rods that serve as electrodes. Opposite rods are connected electrically, one pair being attached to the positive side of a variable dc source and the other pair to the negative terminal. In addition variable radio-frequency ac voltage, are 180 out of a phase, are applied to each pair of rod. To obtain a mass spectrum with this device ions are accelerated into the space between the rods by a potential difference 5 to 10 V. Mean while ,the ac and dc voltage on the rods are increased simultaneously while maintaining their ratio constant. At any given moment, all of the ions except those having a certain m/z value strike the rods and are converted to neutral molecules. Thus only ions having a limited range of m/z values reach the transducer.

To understand the filtering capability of a quadrapole, we need to consider the effect of the dc and ac voltages on the trajectory of ions as they pass through the channel between the rods. In absence of a dc voltage, ions

in the channel will tend to diverge during the negative half. If during the negative held cycle an ion strikes the rod, the positive charge will be neutralized, and the resulting molecule will be carried away. Weather positive ions strikers the rod depends on the rate of movement of the ion along the axis, its mass to charge ratio, and the frequency and magnitude of the ac signal [1].

Time of Flight

In time of flight (TOF) instruments, positive ions are produced periodically by bombardment of the sample with brief pulses of electrons, secondary ions or laser generated photons. These pulses typically have a frequency of 10 to 50 kHz and a life time of 0.25ms. The ions produced in this way are then accelerated by an electric field pulse that has the same frequency as, but lags behind, the ionization pulse. The accelerated particles pass into a field free drift tube about a meter long. Because all ions entering the tube ideally have the same kinetic energy, their velocities in the tube must vary inversely with their masses, with the lighter particles arriving at the detector earlier than the heavier ones. Typical flight times are in the microseconds range for a 1-m flight tube [1].

DETECTORS

Electron multiple

The avalanche can be triggered by any charged particle hitting the starting electrode with sufficient energy to cause secondary emission. Hence the electron multiplier is often used as an ion detector. It could also be triggered by a photon causing vacuum photoemission of at least one electron. In a photomultiplier tube, a photo – emission surface is followed by an electron multiplier with several sequential multiplying electrodes called dynodes. Because these electrodes are separate from each other, this might be called a "discrete dynode" multiplier. A voltage divider chain resistance is usually used to place each dynode at a potential 100v-200 more positive than the previous one A "continuous –dynode" structure is feasible if the material of the electrode has a high resistance so that the functions of secondary –emission and voltage –division are merged. This is often built as a funnel of glass coated inside with a thin film of semi-conducting material, with negative high voltage applied at the wider input end, and appositive voltage near ground applied at the narrower output end. Electrons emitted at any point are accelerated a modest distance down the funnel before impacting the surface, perhaps on the opposite side of the funnel. At the destination end a separate electrode (anode) remains necessary to collect the multiplied electrons. This structure is also known as (single) channel electron multiplier (CEM), and one of the most common is sold under the trade name Channeltron.

Faraday Cup

When a beam or packet of ions hits the metal it gains a small net charge while the ions are neutralized. The metal can then be discharged to measure a small current equivalent to the number of impinging ions. Essentially the faraday cup is part of a circuit where ions are the charge carriers in vacuum and the faraday cup is the interface to the solid metal where electrons act as the charge carriers. By measuring the electrical current (the number of electrons flowing through the circuit per second) in the metal part of the circuit the number of charges being carried by the ions in the vacuum part of the circuit can be determined.

Tandem Mass

The sample is introduced into a ionization source. The ions are then accelerated into Quadrupole 1, which is an ordinary quadrupole mass filter. The selected fast-moving ions pass into quadrupole 2, which is a collision chamber where dissociation of the ions selected by quadrupole 1 occurs. The quadrupole is operated in a radio-frequency –only mode in which no dc voltage is applied across the rods. This mode basically traps the precursor and product ions in a relatively high concentration of collision gas so that CAD can occur. Quadrupole 3 then allows mass analysis of the product ions formed in the collision cell.

Recorders

Direct writing recording oscillograph with 3-5 galvanometer whose relative sensitive are 1, 3, 10, 30, 100 are used for recording purpose. They are very cheap, easy to operate and reliable but need manual manipulation for counting mass number.

Vacuum Systems

Vacuum system is essential right from the sample inlet systems to detector for the operation of any mass spectrometer since it depends on the stream of gaseous ions. Under normal operating conditions, a pressure of about $1* 10^{-7}$mm Hg is maintained and the pressure should not exceed $1*10^{-5}$torr because of the rapid failure of filaments in ionization chamber. Hence, to maintain the required pressure, continuous and efficient pumping of air should be done. For this purpose, diffusion pump backed by a rotary pump is incorporated in mass analyzer and sample inlet systems.

Coupling of CE To ICP-MS

CE is coupled with ICP-MS by sheath flow interface *figure 9.* The attributes of interface are good transport of sample of plasma, ease to use. In this interface a PFA micro flow concentric nebulizer is used to generate an aerosol from the sample. The separation capillary is held with the nebulizer tube via a Teflon chromatographic union with space between the separation capillary and the inner capillary of the nebulizer to allow passage of a

coaxial sheath electrolyte. At typical sheath flow rate ragging from 20 to 100µl/min for the high efficiency PFA micro flow nebulizer using a 50µm i.d. fused silica capillary, this extra column dead volume is swept out in only 0.1 to 1 sec depending on the sheath flow rate.

The inlet end of the separation capillary begins in cheminart Teflon cross. The capillary begins in a cheminart Teflon cross. The capillary exist the cross passes through a sealed peek sleeve. Since the micro flow nebulizer has its own capillary a zero dead volume. PTEE union was used to connect the CE capillary to the nebulizer. A platinum wire was connected to one site of the cross to provide ground which shares the common ground with the CE instrument. A syringe pump was used to deliver the sheath electrolyte from a 10ml glass syringe. Sample together with the electrolyte from both the capillary and the sheath flow were introduced into the plasma as aerosols through the PFA micro-concentric nebulizer and a PFA spray chamber. The choice of a platinum wire and the use of cheminart cross were to reduce the metal background count in the mass spectroscopy. Because the electrolyte solution is flowing throughout the system and is in intimate contact with the effluent from the separation capillary, a stable ground connection to the solution at the end of the separation capillary is achieved [16].

Fig 1. Schematic picture of the instrumental set-up for capillary electrophoresis

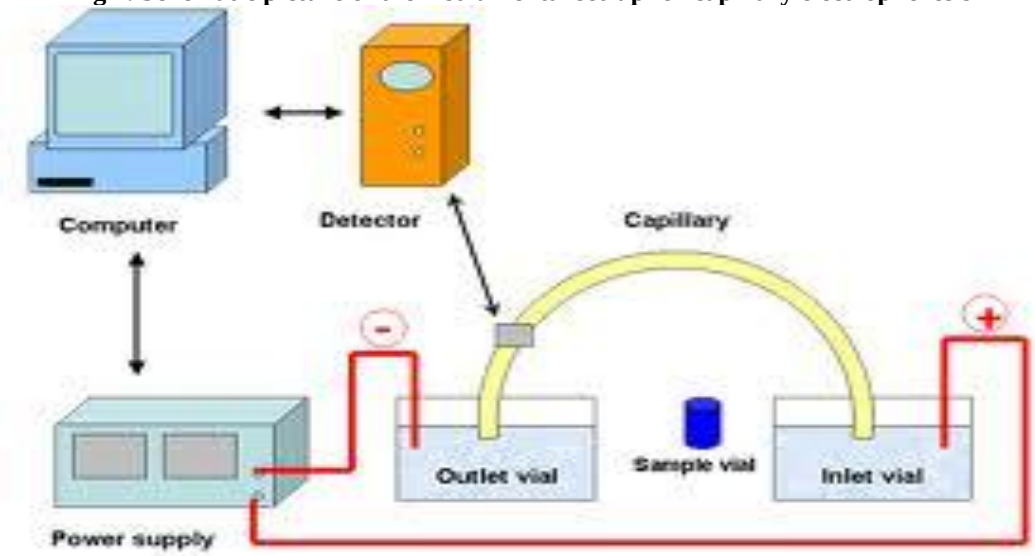


Fig 2. Schematic representation of cross section of capillary of capillary electrophoresis

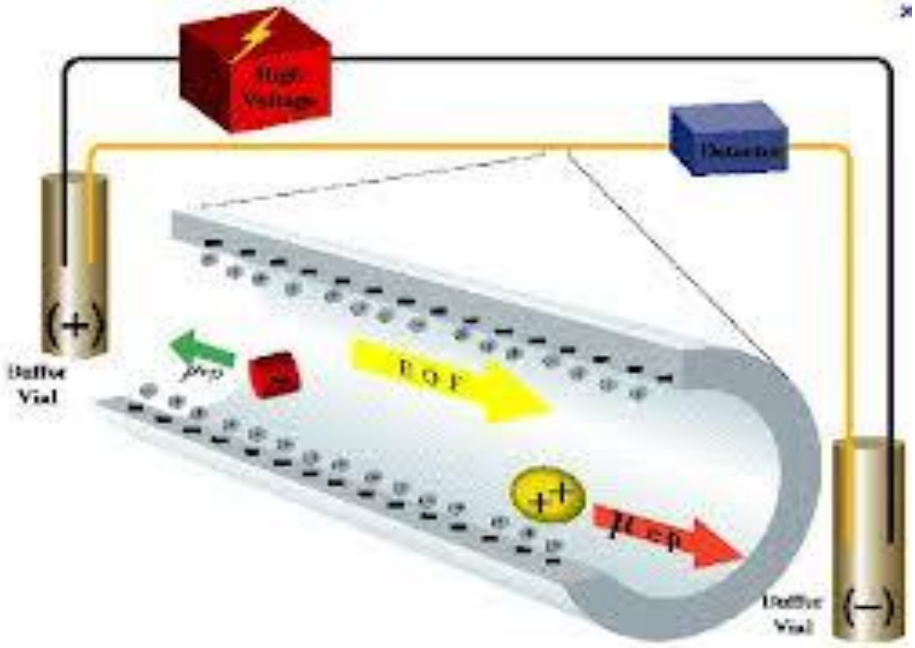


Fig 3. Charged fused silica surface (Si-O-)

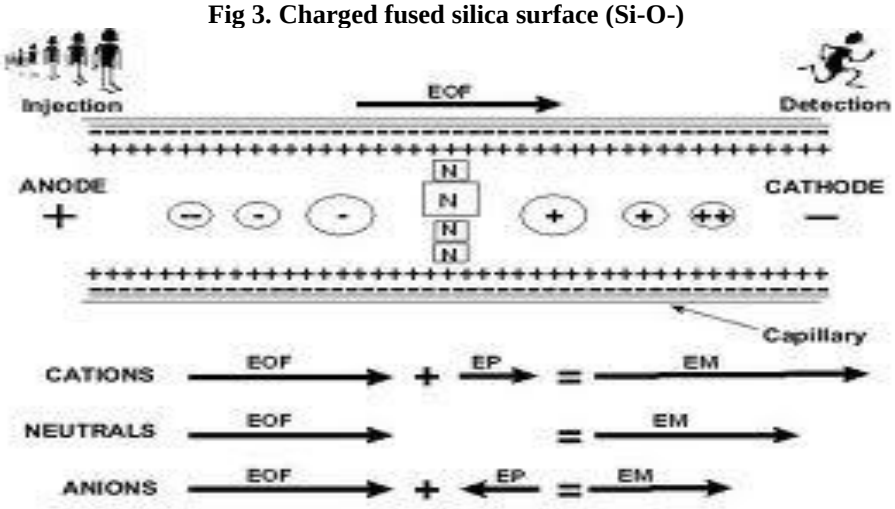


Fig 4. Plasma torch schematic representation

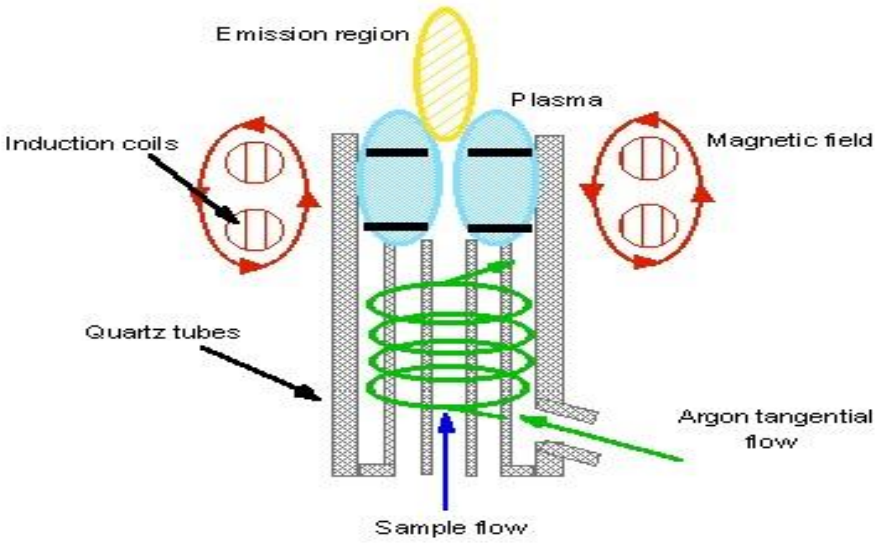
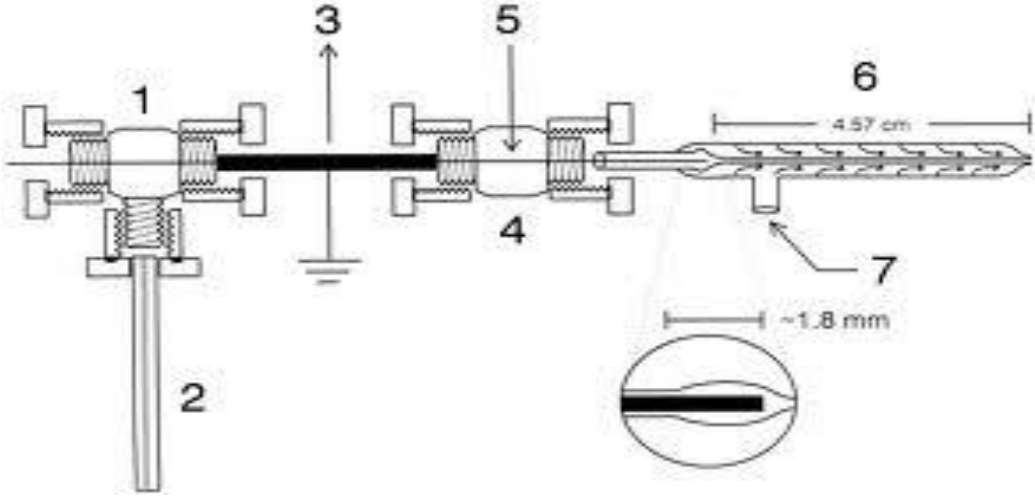


Fig 5. Schematic representation of the sheath flow interface of CE-ICP-MS



1. Teflon cross, 2.syringe pump, 3.platinum tubing with ground, 4.teflon union,
5. fused silica CE separation capillary, 6.PFA micro flow nebulizer,7. Argon gas inlet

APPLICATIONS

- CE-ICP-MS is generally used in studies of metallodrug protein binding studies. Characterization of interaction between pt(II) complexes and human serum albumin in case of anticancer drug like cisplatin [13].
- Investigation of a liposomal oxaliplatin drug formulation by capillary electrophoresis hyphenated to inductively coupled plasma mass spectrometry [19].
- Quantification of phosphorus in DNA using capillary electrophoresis hyphenated with inductively coupled plasma mass spectroscopy [7].
- Characterization of interaction between organotin compound and human serum albumin by capillary electrophoresis coupled with inductively coupled plasma mass spectroscopy [13].
- Taking advantage of the high sensibility of icp-ms for detecting phosphorus this method enabled to assess the affinity of a variety of phosphorylated compound like phosphine oxide, thiophosphine in less than 1hour and using less than 5ng of substance [11].
- Characterization of selenium compounds and separation of different selenium species [15].
- Elucidation of interaction of anti cancer ruthenium complex in clinical trials with biomolecule utilization CE-ICP-MS [12].

CONCLUSION

A good separation strategy before mass spectrometric detection is crucial in a study involving the understanding of complex mixtures such as biological samples in proteomics profiling. This is due to that the good separation also provides good conditions to increase the confidence of protein identifications and to improve the coverage of proteomes. In recent years, many newseparation approaches have been applied successfully in proteome analyses and have provided great improvements in protein identification, although complete coverage of proteins in complex samples has not been achieved. The development of new methods is thus important in order to meet the analytical challenges represented by the huge dynamic range of concentrations and the complexity in biological samples. This thesis describes the CE-ICP-MS contribution to this field & for improved separation performance of proteins in CE when coupled to MS. With a simple change in ionization source in MS we can analyze metallic drug protein complexes. Finally, the thesis describes application of CE in quantitative proteomic analysis with ICP-MS.

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