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Review Article

Cavity Ring Down Laser Absorption Spectroscopy For Evaluation Drug Molecule

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Abstract: A multi wave length, multicomponent CRDS gas sensor operating on the basis of a compact photonic crystal fibre super continuum light source has been constructed. It features a simple design encompassing one radiation source, one cavity and one detection unit (a spectrograph with a fitted ICCD camera) that are common for all wavelengths. Multicomponent detection capability of the device is demonstrated by simultaneous measurements of the absorption spectra of molecular oxygen (spin-forbidden b-X branch) and water vapor (polyads 4v, 4v + δ) in ambient atmospheric air. Issues related to multimodal cavity excitation, as well as to obtaining the best signal-to-noise ratio are discussed together with methods for their practical resolution based on operating the cavity in a "quasi continuum" mode and setting long camera gate widths, respectively. A comprehensive review of multi wave length CRDS techniques is also given.

Keywords: Molecular Spectroscopy, Cavity Ring-Down Absorption, Spectroscopy, Cavity-Enhanced Absorption Spectroscopy.

Introduction

Cavity ring-down spectroscopy (CRDS), also known as cavity ring-down laser absorption spectroscopy (CRLAS), is a laser absorption technique which takes advantage of the improved analytical sensitivity that is possible when making absorption measurements using extremely long path lengths. Effective path lengths of up to a few kilometers can be achieved by using two high-reflectivity mirrors (R>99.9%) to literally "bounce" a pulsed laser back and forth through the absorbing species on the order of about 10,000 times. The light that is transmitted through the exit mirror on each pass is measured vs. time. Under certain conditions, the resulting signal will decay exponentially with time. The decay time will be dependent upon the reflectivity of the two mirrors, the distance between the two mirrors, the speed of light, and the molecular absorption coefficient of any absorbing species in the cavity[1]. Using a tunable pulsed laser source, the decay time (τ) can be recorded as a function of laser wavelength to acquire an absorption spectrum. The unique advantage to this type of measurement is that τ is independent of the initial laser intensity. This translates to improved sensitivities as a result of the removal of signal dependence on shot-to-shot laser variability, a significant source of error in most conventional laser absorption methods for the detection of trace gas species.

Separations science:

In environmental analysis, capillary GC is the benchmark separation technique. For liquid separations a (4.6 mm ID) HPLC column packed with C18-derivatized silica carried out under reverse phase conditions is commonly used. Capillary GC methods are generally very robust with highly reproducible retention times and responses. They can often resolve more than 100 compounds per analysis, resulting in versatility and high peak capacity. However, typical environmental analysis exhibits the resolution of only 10-30 peaks although 1000's of compounds might be involved in the sample. In view of the rather limited peak capacity of one-dimensional separations, it is not surprising that more powerful separation approaches are sought.

LIF is particularly useful in facilitating detection in smaller ID formats such as used in capillary separations (<1.0 µm ID). The detector cell volume generally decreases by the same scaling factors as do all volume-related parameters, i.e. as a square of the ratio of the two diameters (de/ds) 2, but the concentration detection limit appears to follow an approximate simple linear ratio (de/ds) relation or path length where de is the end diameter and ds is the starting diameter 3. For example, assuming that a 4.6 mm ID column system used a 10 µL volume detector cell, then a 0.075 mm ID capillary requires a 2.7 nL cell volume. The obvious advantage of the laser over conventional discharge lamps is the dual ability to focus intense radiation at the wavelength of interest and to do so for a detection cell volume that is the interior diameter of the capillary itself for on-column detection. A typical schematic of an optical bench setup for CE/LIF is shown in Fig (1)

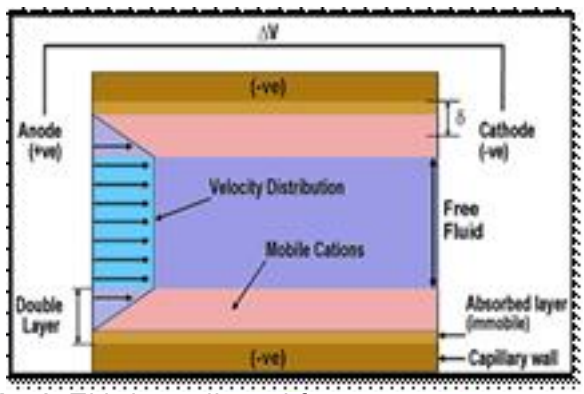

Fig. 1: This is predicated for process

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Fig.2: This is predicated for injecting sample

This schematic illustrates the essential parts of the CE/LIF experiment that consists of the buffers, separation capillary column, excitation light (laser), and the detection of the fluorescent emitted light.

Capillary format separations:

It is obvious that there is a strong trend in analytical chemistry toward developments that address research problems in biomedical areas. This has resulted in the development of tools that determine levels of nonvolatile analytes such as polar drugs, proteins, DNA, and biomarkers[5]. These developments have been partially propelled by capillary format separations such as capillary zone electrophoresis (CZE), capillary electro chromatography (CEC), and the ability of mass spectrometry to ionize large bimolecular via electro spray ionization.

Capillary format separations have an obvious advantage for pollution prevention (low volume of solvent use for separations) and therefore provide a A green@ chemistry approach to analysis. They also exhibit high mass sensitivity (amount of sample on-column that can be detected), but a concomitant increase in concentration detection limits due in one sense to the path length limitations described and the practical limits to the volume of sample that may be injected. This relatively high concentration detection limit is a primary concern for environmental analysis where normally large amounts and volumes of samples are available. Typical detection limits ranging from mg/kg to ng/kg are often realized in commonly used techniques such as GC/FID, GC/ECD, GC/MS, and HPLC/DAD or HPLC/FLD, either directly from a solution of the sample or sample extract or by using pre concentration. A useful figure for target analyte amount for sample injection is 1 pg / µL that is currently practical for GC/MS methods and 1 fg/nL for CE methods. Capillary format separations are, in a practical sense, limited by the volume of injection possible, but this deficiency is offset by the advantage of a plug-like flow profile which results in sharper peaks and greater selectivity[6] from the rather limited practical range of ion mobility. In the case of optical methods of detection, the optical path length is also limited by the geometry of the capillary in any practical detection scheme. Recent work in addressing the short optical path length has been quite creative. A Az-cell@ introduces a straight detour into the inside of the capillary, allowing the laser to interact with the contents along a section of the capillary length rather than through a cross-section.

LIF has assumed an important role as a detector for CZE and other capillary techniques because of its inherent sensitivity and resultant low detection limits (typically, 10-7 M to 10-13 M). Ultraviolet detection methods are unable to achieve these low detection limits, typically being limited to about 10-6M-10-7M. For organic ions, the power of LIF increases our ability to sensitively screen for nonvolatile and other analyses[7].

Derivatization of target analytes with fluorophores:

Since there is a requirement for the target analyte to exhibit fluorescence, some analysts have resorted to derivatization with fluorophores to provide the desired properties. In principle, this approach would produce a derivative analyte capable of being detected at very low levels.

Two limiting considerations must be addressed within this context, however. One concerns the lowest practical concentration of analyte that will undergo reaction with the derivatizing reagent[8]. Chemical kinetics dictates that some analytes will not react appreciably at concentrations of interest, but must first be pre concentrated in order for the reactions to proceed at useful rates.

The second consideration must address the production of artifacts, coupling products, and derivatives from co-extractives, all of which put additional burden on the separation system. To address these very challenging problem two approaches may be pursued. First, one may apply cleanups to limit the number of co-extractives present. Second, one may develop cleanups to remove the bulk of the unwanted side products and other fluorescing background from the reaction mixture[9].

Quality Assurance/Quality Control (QA/QC) aspects of CE/LIF:

Two primary questions arise in the context of QA/QC issues: How reproducible are the run to run migration times of target analytes? How transferable and robust are methods developed in one laboratory when ported to another laboratory (or collaborative study)? Unfortunately, the performance to date for both CZE and micelle conditions has not been as good as those methods based on GC/MS, HPLC, and TLC.

The well known variation in electro osmotic (EO) flow has been a major source of irreproducibility. One QA/QC tool that has been used is to employ an internal standard as both a quantitative tool and as a corrective tool for migration time variations, especially when those variations are a result of EO flow variations[10]. Migration time (MT) corrections can be applied based on the reciprocal relation between intrinsic mobility and MTs. Thus, if a typical standard run is used as a benchmark for the MTs of internal standard and analytes, subsequent runs of samples can be corrected back to this benchmark where the correction factor is based on the relationship between the MT of the internal standard in the two runs. This factor can then be used to correct the analyte MTs to what they would be if the EO flow was the same as the benchmark run. Typically, corrected MTs of analytes can be as good as 0.3 % reproducibility.

A more difficult problem to deal with is the change in selectivity that is observed when methods are transferred to a different laboratory. Separations can no longer be maintained, or the order of analytes that reach the detector is altered[11]. The reproducibility issue is particularly acute under micelle conditions. Adsorption problems also occur in free zone, particularly when non-borate buffers are used or when dealing with ions that are subject to these problems.

Corrected peak areas must be used when on-column detection is implemented. This is usually automatically handled by the data system software, but the analyst may have to factor this in for laboratory built systems[12]. This is a direct result of the difference in time that the analyte spends within the detector window as a function of its apparent mobility as it moves through the capillary.

It is considered good practice to bracket the sample series of runs with standards, validating performance and adherence to the calibration plot. Analysis of reagent blanks to demonstrate the lack of contamination and solvent blanks between samples and standards is also recommended[13].

Environmental analysis:

A number of examples will illustrate the attributes and advantages of CE/LIF for solving the analytical problems posed by various environmental analysis scenarios. The performance or potential of CE/LIF will be compared with the currently used methodology shown in fig (2)

Dye tracers in groundwater migration studies: Groundwater migration using fluorescent dyes presents an analytical problem almost ideally matched to CZE/LIF. The anionic dyes migrate in such a manner that they follow the cations and neutrals in entering the detection window (longer MTs). CZE/LIF has been used for tin opal (near UV, 354 nm excitation) and fluoresce in (visible, 488 nm excitation) dyes in actual groundwater migration. These applications may result in improved detection limits, specificity, and quality control. Multi-wavelength lasers may be applied for multiple dye injection studies. Typical dyes used include fluoresce in, tin opal (fluorescent brightener), eosin, and rhodamines[14].

Solid waste and contaminants in solid matrices: It is possible to screen for a wide range of analytes that fluoresce using frequency-doubled lasers operating in the deep UV (e.g., 257 nm). Alkyl phenols and hydroxyl-PNAs can be detected as migrating anions and therefore separated from neutral hydrophobic[15].

Micellar agents or cyclodextrins offer alternatives and are needed for the resolution of neutral molecules using a technique called micellar electro kinetic chromatography (MECC). This is accomplished for PNAs based on separations using cyclodextrins with LIF detection resulting from the HeCd laser operated at 325 nm.

Atmospheric contaminants: Capillary electrophoresis has been applied to various substances of interest to atmospheric chemistry and particulate matter. Recent work by Dabek-Zlotorzynska, shows that CE/LIF can be used to measure dim ethylamine and other low-molecular-weight amines in atmospheric aerosol studies[16].

Chemical characterization of matrices:

The characterization of a sample can be limited to a list of analytes of interest or it can be expansive enough to encompass the character of the matrix itself. Humic substances include fulvic acid, humic acid, and humin. These constituents, while not frequently considered in environmental analyses, play an important role in the way contamination binds to or flows through soil matrices[17]. CE has been used to study the interaction of free metal cations with fulvic acid. It has also been used to characterize sewage effluent for fluorescent acids.

Drinking water and groundwater contaminants:

An area that has stimulated considerable recent interest both publicly and analytically is that of endocrine disrupting compounds (EDCs). There is a demand for reliable analytical methods capable of detecting trace levels of many pesticides, polychlorinated biphenyls, and dioxin-like compounds, among others. CE/LIF may play a role in applicable cases providing some type of fluorescent property belongs to the target or can be added to the target analyte[18]. This focus on EDCs has resulted in closer scrutiny of many pesticides

and other suspect compounds such as polychlorinated biphenyls. The Safe Drinking Water Act (SDWA) and the Food Quality Protection Act (FQPA) have both adopted new regulations for monitoring thousands of compounds that interfere with the human and ecological hormone systems[19].

An example of the application of CE to pesticide analysis is the use of MECC with LIF to analyze for trace levels of phenoxy acid herbicides. This highly sensitive method requires a complex derivatization but is able to achieve femtomole detection levels. CE even has the power and sensitivity to separate enantiomers of phenoxy acid herbicides. CE/LIF in an immunoassay format was also applied to 2, 4-dichlorophenoxy acetic. CE/LIF has been used to determine anilines and aliphatic amines. Surfactants have also been determined using CE/LIF operating in the UV region.

Biomarkers of exposure:

Biomarkers of exposure can be monitored by analyzing certain physical response parameters such as protein adducts, DNA adducts, and other biological indicators[20]. This application seeks to exploit the sensitivity and specificity of LIF and the separation power of CE. However, the area has yet to undergo significant development.

Food contaminants:

The complexity of food analysis is a combination of difficult matrices and low detection level requirements. In addition, the contaminants present in food may be pesticide residues, natural toxins, such as mycotoxins, or the residues or breakdown products of food additives. CE with fluorescence detection has been used to determine the levels of fumonisin B1. Certain food contamination issues have become matters of widespread interest to the media and the public. The low detection limits achieved by CE/LIF make it an excellent choice for food analysis and monitoring where applicable.

MECC with LIF has been used in a study of aflatoxin contamination in corn. In a laboratory study, aflatoxin spores were introduced onto corn kernels and allowed to grow for two weeks at room temperature[21]. The complex sample matrix yielded chromatographic peaks that were difficult to resolve. MECC data showed separation times of less than a minute and, at the time of the study, the detection was the limiting factor.

Emerging developments:

Multidimensional separation:

Multidimensional separations allow more complete analyses because analytes are separated by more than one method, e.g., GC and (LC), supercritical fluid extraction (SFE) and LC, or other combinations. Though in theory, less efficient separation is obtained with two-dimensional chromatography, the advantage in separation power greatly outweighs any loss in efficiency. The resolution obtained depends on several factors: the orthogonality of the methods, the effectiveness of transferring from one column to another, and the completeness of the whole sample dispersion[22].

CZE has been used with reversed-phase HPLC in an automated comprehensive two-dimensional method. Each separation phase is capable of effecting separations using a different separating principal: they are orthogonal methods. Bushy and Jorgenson presented the advantages (excellent separation, ease of injection into the CZE system) and disadvantages (long analysis time, inefficiency in sampling from the first column).

CEC has been used to separate 16 different polycyclic aromatic compounds (PAHs). In CEC, an electric field is applied across columns that are packed with micro particulates. The electro osmotic flow becomes a tool for chromatographic separations. CEC has an advantage over CZE in this application because it is capable of separating many uncharged species[23].

Instrumental Setup:

A typical capillary electrophoresis system consists of a high-voltage power supply, a sample introduction system, a capillary tube, a detector and an output device. Some instruments include a temperature control device to ensure reproducible results. This is because the separation of the sample depends on the electrophoresis mobility and the viscosity of the solutions decreases as the column temperature rises. Each side of the high voltage power supply is connected to an electrode. These electrodes help to induce an electric field to initiate the migration of the sample from the anode to the cathode through the capillary tube. The capillary is made of fused silica and is sometimes coated with polyimide. Each side of the capillary tube is dipped in a vial containing the electrode and an electrolytic solution, or aqueous buffer. Before the sample is introduced to the column, the capillary must be flushed with the desired buffer solution[24]. There is usually a small window near the catholic end of the capillary which allows UV-VIS light to pass through the analyte and measure the absorbance. A photomultiplier tube is also connected at the cathodic end of the capillary, which enables the construction of a mass spectrum, providing information about the mass to charge ratio of the ionic species in fig(3)

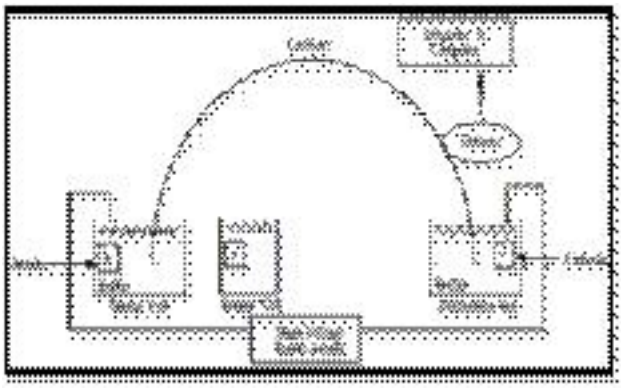

Fig. 3: This is predicated for set up

Theory:

Electrophoresis Mobility: Electrophoresis is the process in which sample ions move under the influence of an applied voltage. The ion undergoes a force that is equal to the product of the net charge and the electric field strength. It is also affected by a drag force that is equal to the product of f, the translational friction coefficient, and the velocity. This leads to the expression for electrophoretic mobility:

$$\mu EP = q / f = q / (6\pi\eta r)$$

Where f for a spherical particle is given by the Stokes' law; η is the viscosity of the solvent, and r is the radius of the atom. The rate at which these ions migrate is dictated by the charge to mass ratio. The actual velocity of the ions is directly proportional to E, the magnitude of the electrical field and can be determined by the following equation:

$$v = \mu EP * E$$

This relationship shows that a greater voltage will quicken the migration of the ionic species.

Electroosmotic Flow:

The electro osmotic flow (EOF) is caused by applying high-voltage to an electrolyte-filled capillary. This flow occurs when the buffer running through the silica capillary has a pH greater than 3 and the SiOH groups lose a proton to become SiO- ions. The capillary wall then has a negative charge, which develops a double layer of cations attracted to it. The inner cation layer is stationary, while the outer layer is free to move along the capillary. The applied electric field causes the free cations to move toward the cathode creating a powerful bulk flow. The rate of the electro osmotic flow is governed by the following equation:

$$\mu EOF = \varepsilon/4\pi\eta * E\zeta$$

where ε is the dielectric constant of the solution, η is the viscosity of the solution, E is the field strength, and ζ is the zeta potential. Because the electrophoresis mobility is greater than the electro osmotic flow, negatively charged particles, which are naturally attracted to the positively charged anode, will separate out as well. The EOF works best with a large zeta potential between the cation layers, a large diffuse layer of cations to drag more molecules towards the cathode, low resistance from the surrounding solution, and buffer with pH of 9 so that all the SiOH groups are ionized.

Electro osmotic Flow due to Applied Voltage:

Capillary Electro separation Methods: There are six types of capillary electro separation available: capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), micellar electro kinetic capillary chromatography (MEKC), capillary electro chromatography (CEC), capillary isoelectric focusing (CIEF), and capillary isotachophoresis (CITP). They can be classified into continuous and discontinuous systems as shown in Figure 3. A continuous system has a background electrolyte acting throughout the capillary as a buffer. This can be broken down into kinetic (constant electrolyte composition) and steady-state (varying electrolyte composition) processes 26. A discontinuous system keeps the sample in distinct zones separated by two different electrolytes.

Capillary Zone Electrophoresis (CZE):

Capillary Zone Electrophoresis (CZE), also known as free solution capillary electrophoresis, it is the most commonly used technique of the six methods. A mixture in a solution can be separated into its individual components quickly and easily. The separation is based on the differences in electrophoresis mobility, which is directed proportional to the charge on the molecule, and inversely proportional to the viscosity of the solvent and radius of the atom. The velocity at which the ion moves is directly proportional to the electrophoresis mobility and the magnitude of the electric field.

The fused silica capillaries have silanol groups that become ionized in the buffer. The negatively charged SiO- ions attract positively charged cations, which form two layers—a stationary and diffuse action layer27. In the presence of an applied electric field, the diffuse layer migrates towards the negatively charged cathode creating an electrophoretic flow (μep) that drags bulk solvent along with it. Anions in solution are attracted to the positively charged anode, but get swept to the cathode as well. Cations with the largest charge-to-mass ratios separate out first, followed by cations with reduced ratios, neutral species, anions with smaller charge-to-mass ratios, and finally anions with greater ratios. The electro osmotic velocity can be adjusted by altering pH, the viscosity of the

solvent, ionic strength, voltage, and the dielectric constant of the buffer in fig (3).

Capillary Gel Electrophoresis (CGE):

CGE uses separation based on the difference in solute size as the particles migrate through the gel. Gels are useful because they minimize solute diffusion that causes zone broadening, prevent the capillary walls from absorbing the solute, and limit the heat transfer by slowing down the molecules. A commonly used gel apparatus for the separation of proteins is capillary SDS-PAGE. It is a highly sensitive system and only requires a small amount of sample fig (4).

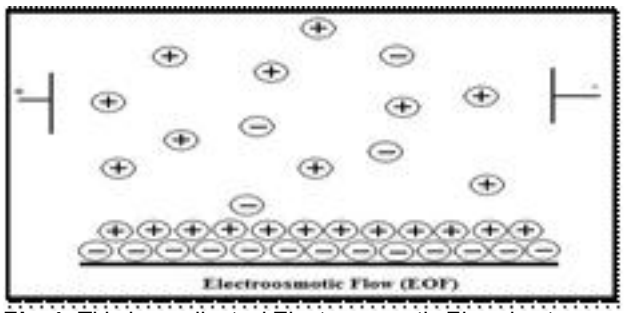

Fig.4: This is predicated Electro osmotic Flow due to Applied Voltage

Micellar Electro kinetic Capillary Chromatography (MEKC):

MEKC is a separation technique that is based on solutes partitioning between micelles and the solvent. Micelles are aggregates of surfactant molecules that form when a surfactant is added to a solution above the critical micelle concentration. The aggregates have polar negatively charged surfaces and are naturally attracted to the positively charged anode. Because of the electro osmotic flow toward the cathode, the micelles are pulled to the cathode as well, but at a slower rate. Hydrophobic molecules will spend the majority of their time in the micelle, while hydrophilic molecules will migrate quicker through the solvent[28]. When micelles are not present, neutral molecules will migrate with the electro osmotic flow and no separation will occur. The presence of micelles results in a retention time to where the solute has little micelle interaction and retention time tmc where the solute strongly interacts. Neutral molecules will be separated at a time between two and tmc. Factors that affect the electro osmotic flow in MEKC are: pH, surfactant concentration, additives, and polymer coatings of the capillary wall, in fig (5).

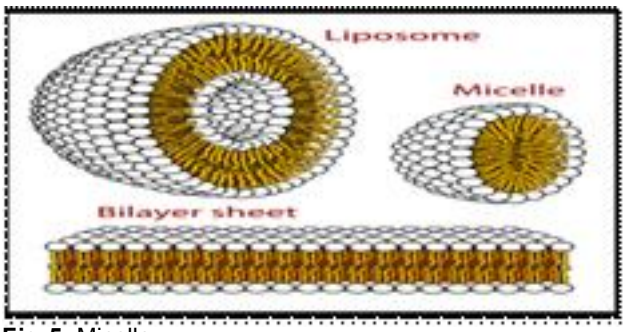

Fig.5: Micelle

Capillary Electro chromatography (CEC):

The separation mechanism is a packed column similar to chromatography. The mobile liquid passes over the silica wall and the particles. An electro osmosis flow occurs because of the charges on the stationary surface. CEC is similar to CZE in that they both have a plug-type flow compared to the pumped parabolic flow that increases band broadening.

Capillary Isoelectric Focusing (CIEF):

CIEF is a technique commonly used to separate peptides and proteins. These molecules are called zwitterionic compounds because they contain both positive and negative charges. The charge depends on the functional groups attached to the main chain and the surrounding pH of the environment. In addition, each molecule has a specific isoelectric point (pI). When the surrounding pH is equal to this pI, the molecule carries no net charge. To be clear, it is not the pH value where a protein has all bases deprotonated and all acids protonated, but rather the value where positive and negative charges cancel out to zero. At a pH below the pI, the molecule is positive, and then negative when the pH is above the pI. Because the charge changes with pH, a pH gradient can be used to separate molecules in a mixture. During a CIEF separation, the capillary is filled with the sample in solution and typically no EOF is used (EOF is removed by using a coated capillary). When the voltage is applied, the ions will migrate to a region where they become neutral (pH=pI). The anodic end of the capillary sits in acidic solution (low pH), while the cathodic end sits in basic solution (high pH). Compounds of equal isoelectric points are “focused” into sharp segments and remain in their specific zone, which allows for their distinct detection.

Calculating pI:

An amino acid with n ionizable groups with their respective pKa values pK1, pK2, .pkn will have the pI equal to the average of the group pkas: pI = (pK1+pK2+...+pkn)/n. Most proteins have many ionizable side chains in addition to their amino- and carboxy- terminal groups. The pI is different for each protein and it can be theoretically calculated according to the Henderson-Hasselbalch approximation, if we know amino acids composition

of protein. In order to experimentally determine a protein's pI 2-Dimensional Electrophoresis (2-DE) can be used. The proteins of a cell lysate are applied to a pH immobilized gradient strip, upon electrophoresis the proteins migrate to their pI within the strip.

Capillary Isotachorphoresis (CITP):

CITP is the only method to be used in a discontinuous system. The analyte migrates in consecutive zones and each zone length can be measured to find the quantity of sample present. Capillary Electrophoresis versus High Performance Liquid Chromatography (HPLC).

1. CE has a flat flow, compared to the pumped parabolic flow of the HPLC. The flat flow results in narrower peaks and better resolution.
2. CE has a greater peak capacity when compared to HPLC—CE uses millions of theoretical plates.
3. HPLC is more thoroughly developed and has many mobile and stationary phases that can be implemented.
4. HPLC has more complex instrumentation, while CE is simpler for the operator.
5. HPLC has such a wide variety of column lengths and packing, whereas CE is limited to thin capillaries.
6. Both techniques use similar modes of detection.
7. Can be used complementary to one another.

Materials and Methods

Chemicals:

All organic compounds were obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA) and Molecular Probes (Eugene, OR) unless otherwise specified. Other chemicals were from standard sources of supply, and all were used as received. Deionized water (18 mohm quality) was used for all aqueous solutions. Buffer solutions were freshly prepared at least weekly. Solutions of dye standards were prepared from solid dye and serially diluted.

Optics and fused silica:

Fused silica glass with polyimide coating was obtained from PolyMicro Technology, Phoenix, AZ, USA. Capillaries of 0.050 mm ID and 0.075 mm ID are acceptable for separations. Optics obtained commercially should be appropriate for the application. If wavelengths below 365 um are used, then fused silica optics should be employed with appropriate coatings for the application wavelength region.

Capillary electrophoresis of fluoresce in dyes:

A P/ACE Model 5000 Capillary Electrophoresis System (Beckman Instruments, Fullerton, CA, USA) was used for all commercial instrument electrophoresis determinations reported here. The instrument was fitted with a capillary 57 cm in total length (50 cm from the origin to the detector window) and 75 µm inside diameter. Detection was accomplished with an Ar ion laser operated at 488 nm emission and detection using a notch filter (488 nm) and a band pass filter (520DF20). Unless otherwise noted, electrophoresis was carried out at 30 kV. The temperature of the capillary was maintained at 25EC. The capillary was equilibrated with running buffer for two minutes prior to beginning of an experiment, and washed for two minutes with alkali and water between runs. Migration times, peak widths, and peak areas were determined directly from peaks displayed by the data system or by processing software. Corrected peak areas, as computed by the instrumental software were further normalized by dividing them by the area of the peak of the internal standard, which was erythrosine B. This computation was necessary to correct for the small variations in injection volumes resulting from the pressure injections typically of 5 sec duration (nominally 20 nL).

Sample handling:

Samples containing dyes or other fluorophores can be pre-concentrated from the matrix by several procedures.

Calibration Curve:

A regression analysis was carried out on the ratio of corrected areas (fluoresce in corrected area divided by internal standard corrected area) versus the ratio of fluoresce in concentration to internal standard concentration from 1 X 10-7 M to 1 X 10-10 M in half decade increments resulting in a 7-point calibration curve. Both unforced and forced through the origin regressions were considered and both resulted in correlation coefficients of 0.99. The equation of the line was used to calculate the concentrations of fluoresce in the samples and check standards run during the course of analysis. The concentration of fluoresce in was calculable based on the known volume and concentration of internal standard added to the sample and the known volume/weight of the sample being analyzed.

Quality Assurance/Quality Control (QA/QC):

Each group of samples to be analyzed was bracketed before and after by a representative standard/internal standard QC sample to establish adherence to the calibration curve equation and MT variations due to changes in EO flow and therefore MT. Deviations 15% in agreement greater than with the calibration curve results would be cause for rerunning of standards, construction of a new calibration curve, or replacement of the capillary.

The migration time variation on a given day/capillary was 5% in agreement with the standards of approximately that day. The variation provides a rough window for anticipating the response of the internal standard and fluoresces in. Using MT correction based on the internal standard, the expected and measured MT of fluoresce in peak fell within 0.3%. The position of both the internal

standard and fluoresce in could also be estimated from the position of the EO flow disturbance. The EO flow could be seen as a peak on the optical scale or can be monitored as a change in current as the injection plug exits the column. Further confirmation of the internal standard peak can be obtained by over spiking the sample with an additional aliquot of internal standard and observing the appropriate increase in peak area. Fluoresce in itself could be confirmed by a similar procedure. In the samples reported in this work, no problems were encountered in identifying internal standard and analyte responses due to the low background level observed with CZE/LIF.

Optical bench experiments with optical brighteners:

An optical bench with components and light-tight enclosure was constructed and used for all LIF experiments. The overall design was based on that of Nye, but modified in certain respects. A special cylindrically symmetric capillary holder was machined to insure optical alignment with the laser beam, lenses, slit, and the newly installed capillary with window made by removing the polyimide coating. The entrance lens was an L-50X and the exit lens was an M-60X (Newport, Irvine, CA). A mass spectrometer slit (VG 7070EQ source slit, Micro mass, Beverly, MA) was mounted in a special holder to exclude wall fluorescence transmitted to the detector as previously described. The bench was fitted with a fused-silica m I.D., 50 cm capillary Polymicro Technologies, Phoenix, AZ) 57 cm X 75- to the detector, with LIF detection using the 354-nm line of the HeCd ion laser, 3 mW, model 7203N (Liconix, Santa Clara, CA) and two 450DF100 (i.e., 50% transmission at 400 nm and 500 nm) emission filters (Omega Optical, Brattleboro, VT) in series. The power supply for CE was a Series EL (Glassman, White House Station, NJ). The temperature of the capillary was 25°C, and electrophoresis runs were about 10 minutes at 20 kV using a 40 mM borate buffer at pH 9.1. The buffer was prepared by weighing 0.381 g of sodium tetraborate decahydrate followed by dissolution in 100 mL of deionized (DI) water. The capillary was equilibrated with running buffer at the start of each experiment, and washed extensively (minimum 2 min each) with 0.1 M sodium hydroxide, DI water, and running buffer between analyses. Rinsing was accomplished by using capillary rinse reservoirs (SGE, Austin, TX) at about 20 psi of nitrogen pressure by fitting the injection end of the capillary through septum seals on the reservoirs. Migration times, peak widths, and detection limits were either read directly from the monitor or from printouts of the data system (Austin P-90 computer, Austin, TX loaded with Beckman System Gold, Ver. 8.1, Fullerton, CA) with data acquisition using a Beckman 406 analog interface (2 V full scale output). The photomultiplier tube (PMT) was model R928 (185-900 nm) fitted with socket E0719-21 (Hamamatsu Photonics Systems, Bridgewater, NJ) and was operated at 900 V with power supply model 230-03R (Bertan, supplied by Hamamatsu). The current amplifier for the optical signal was a model 428 (Keithley Instruments, Cleveland, OH) and used the auto-current suppression facility of the amplifier to zero the background signal and maintain full amplifier dynamic range (0 to 10 V output). Corrected peak areas, as computed by using a spreadsheet (peak area multiplied by the velocity of the ion [length to the detector divided by time]), were normalized to the corrected peak area of the internal standard (7-hydroxycoumarin-4-acetic acid) as a control for the variations in the nominal volumes of the gravity injections (10 sec to 40 sec at 30 cm height corresponding to about 40 to 170 nL). A microampere electrometer with 0 to 1 V output (1 V = 200 µA) was constructed for measuring current through the capillary and was also interfaced to the Beckman 406 ADC to provide a record of the electrophoresis current.

Four different dyes (Tinopal CBS-X, fluoresce in (acid yellow 73), rhodamine WT, and eosin Y) were injected into four wells at a RCRA site and were monitored at three wells at a nearby Superfund site. Each dye (10 - 30 lbs) was injected with 2000 L of water resulting in a 10 mM concentration level for each dye. Thereafter, 8000 L of water was used to flush the dyes into the surrounding groundwater. Samples were taken before injection and for about two months afterward resulting in about 22 samples. Samples consisted of vial samples of water, "receptors", and 1-L water samples at the monitoring wells. The "receptors" consisted of fiberglass mesh filled with coconut charcoal and weighted to remain near the bottom of the well. The standard protocol called for 1 g of charcoal from the receptor to be extracted with 10 mL of a solution consisting of 5:3:2 (propanol: water: concentrated ammonium hydroxide). Results for fluoresce in may be reported as ppt-levels in the 10-mL extract ant of the pads or ppt in the water when determined directly from a portion of the water sample.

SPE Sample Handling:

Fluorescein was isolated from spiked DI water samples or groundwater samples using SPE with styrene-divinylbenzene (SDVB) extraction disks. The disks were prepared following the manufacturer's directions by soaking in 10 mL acetone and then pulling the solvent through the disk28. The process was repeated with 10 mL methanol and then water without letting the disk become dry. Samples were then added, adjusted to pH 5.0, and pulled through at 25 mm Hg vacuum. The disks were dried for 2 min and then eluted twice with 6 mL of methanol. The methanol eluant was concentrated as necessary with a gentle stream of

nitrogen with gentle warming to achieve a recovered concentration within the detection limits of the CE/LIF technique (fig 6).

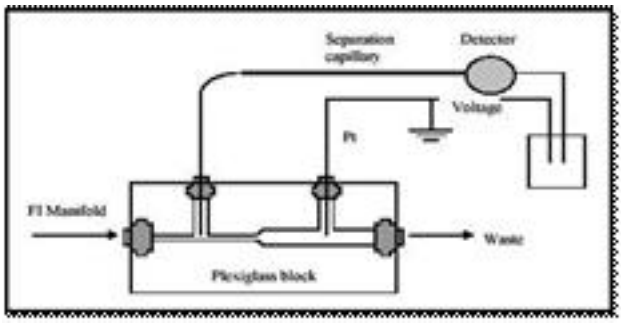

Fig.6: This is predicated for injecting

CE application in biopharmaceutical analysis:

CE has demonstrated to be a complementary alternative to chromatographic techniques in biopharmaceutical analysis. The complete characterization of biopharmaceutical drugs such as erythropoietin and various therapeutic monoclonal antibodies for glycosylation compositions, IgG purity, and impurities for quality control purposes are of utmost importance. Further, therapeutic bimolecular have a highly complex composition and structure. Moreover, the products may vary in structure due to the complexity of cell culture and purification processes 329. Various modes of CE offer several possibilities for biopharmaceutical analysis including glycosylated therapeutic proteins, monoclonal antibodies, pharmaceutical and biopharmaceutical impurities 330. CZE has been used for proteins as long as there are differences in charge: frictional drag ratios 331. The acceleration and rapid success of Human Genome Project was possible due only to the introduction of CE-based sequencers 332. CE-SDS gel application has become the gold standard for protein purity and heterogeneity analysis in biopharmaceutical laboratories and several major biopharmaceutical companies have adopted CE-SDS as a replacement to SDS-PAGE. Denatured proteins can be reduced or left intact for separation and subsequent analysis 333. Various modes of CE offer numerous possibilities for biopharmaceutical analysis 334. CE- laser-induced fluorescence detection method has been useful for both structural characterization and quantitative profiling of Nlinked oligosaccharides derived from recombinant monoclonal antibodies pharmaceuticals 335. CIEF applications related to analysis of biopharmaceutical compounds and isolated proteins for metabolomic studies have been reported 336. A novel online 2-D system combining CIEF with pCEC using a microinjection valve as the interface was developed for peptide and protein mapping. Separation effectiveness of this 2-D system was demonstrated by the analysis of tryptic digest of BSA and human red blood cell lysate. A theoretical peak capacity of approximately 24 000 achieved for bovine serum albumin digest proved its promising potential for the application in proteomics 337. DNA fragments, SDS protein and macromolecules analysis has been achieved using CGE 338-342. Flow Induced Dispersion Analysis (FIDA), a general methodology for assessing non-covalent interactions, has been used on small molecules, proteins (such as antibodies)[30].

CE applications in biotechnology:

CE separation technique is broadly used in the biotechnology industry for carbohydrate analysis and significant improvements for the standard CE sample preparation method of glycan analysis of glycoprotein's by CE-LIF and CE-MS were reported 361-366. AdvanCE™ FS platform provided rapid separation and ample resolution with excellent sensitivity and dynamic range, to benefit a variety of applications in genomic research 367. Several glycoproteins such as fetuin, alpha1 acid glycoprotein, IgG, and transferring separation was achieved within only 5 h with the three-step procedure involving release of glycans, derivatization with Fmoc, and CE-ESI MS analysis. This method was also applicable or the analysis of N-glycans derived from monoclonal.

References

1. Daughton, C. G., Ternes, T. A., (1999) Pharmaceuticals and personal care products in the environment: Agents of subtle change? Environ. Health Perspectives 107(suppl 6), 907.
2. http://www.epa.gov/ppcp/
3. http://www.epa.gov/nerlesd1/chemistry/org-anal/home.htm
4. Brumley, W. C. and Winnik, W. (1996) Applications of Capillary Electrophoresis/Mass Spectrometry to Environmental Analysis in Applications of Liquid Chromatography/Mass Spectrometry in Environmental Chemistry. D. Barceló, ed., Elsevier, Amsterdam, C12, p481-527.
5. Heiger, D. N., (1992) High Performance Capillary Electrophoresis, Hewlett-Packard 12-5091-6199E, Walbronn, Germany.
6. Brumley, W. C. (1995) Techniques for handling environmental samples with potential for capillary electrophoresis. J. Chromatogr. Sci. 33, 670-685.
7. Gooier, C., Kok, S.J., and Ariese, F. (2000) Capillary electrophoresis with laser-induced fluorescence detection for natively fluorescent analytes Analusis, 28, 679-685.
8. Chervet, J. P., van Ling, R., Evans, K., and Salzmann, (1999) J.-P. Capillary and nano HPLC using a dedicated instrument. Am. Lab. August, 44-50.
9. Dadoo, R., Yan, C., Zare, R.N., Anex, D.S., Rakestraw, D.J., and Hux, G.A. (1997) Advances toward the routine use of capillary electro chromatography. LC-GC, 15(7) 630-635.
10. Chervet, J. P., van Soest, R. E. J., and Ursem, M. (1991) Z-Shaped flow cell for UV detection in capillary electrophoresis. J. Chromatogr. 543, 439-449.

11. Nie, S., Dadoo, R. and Zare, R. N. (1993) Ultrasensitive fluorescence detection of polycyclic aromatic hydrocarbons in capillary electrophoresis. Anal. Chem. 65, 3571.

12. Brumley, W. C. and Brownrigg, C. M. (1993) Electrophoresis behavior of aromatic-containing organic acids and the determination of selected compounds in water and soil by capillary electrophoresis. J. Chromatogr. 646, 377-389.

13. Brumley, W. C., Ferguson, P. L., Grange, A. H., Donnelly, J. L., and Farley, J. W. (1996) Applications of capillary electrophoresis/laser-induced fluorescence detection to groundwater migration studies. J. Cap. Electrophoresis 3, 295-299.

14. Ferguson, P. L., Grange, A. H., Brumley, W. C., Donnelly, J. L., and Farley, J. W. (1998) Capillary electrophoresis/laser-induced fluorescence detection of fluoresce in as a groundwater migration tracer. Electrophoresis 19, 2252-2256.

15. Brumley, W. C., and Gerlach, C. L. (1998) Capillary electrophoresis/laser-induced fluorescence in groundwater migration determination. Amer. Laboratory 31, 45-49

16. Skoog, D.A.; Holler, F.J.; Crouch, S.R "Principles of Instrumental Analysis" 6th ed. Thomson Brooks/Cole Publishing: Belmont, CA 2007

17. Brumley, W. C., Grange, A. H., Kelliher, V., Patterson, D. B., Montcalm, A., Glassman, J., and Farley, J. (2000) Environmental screening of acidic compounds based on CZE/LIF detection with GC/MS and GC/HRMS identifications. JAOAC International 83, 1059-1067.

18. Brown, R.S., Loon, J.L.T., Szolar, O., Halasz, H.J., and Hawari, J.A. (1996) Cyclodextrin-modified capillary electrophoresis: Determination of polycyclic aromatic hydrocarbons in contaminated soils. Anal. Chem. 68, 287-292.

19. Yan, C., Dadoo, R., Zhao, H., Zare, R.N., and Rakestraw, D.J. (1995) Gradient elution in capillary electro chromatography. Anal. Chem., 67 2026-2029.

20. Dabek-Zlotorzynska, E., Piechowski, M., Liu, F., Kennedy, S., and. Dlouhy, J. F (1997) Routine determination of major ions in atmospheric aerosols by capillary electrophoresis. J. Chromatogr. A 770, 349-359.

21. Dabek-Zlotorzynska, E. and Maruszak, W. (1998) Determination of dim ethylamine and other low-molecular-mass amines using capillary electrophoresis with laser-induced fluorescence detection. J. Chromatogr. B 714, 77-85.

22. Nordén, M. and Dabek-Zlotorzynska, E. (1996) Study of metal-fulvic acid interactions by capillary electrophoresis J. Chromatogr. A 739, 421-429.

23. Flaherty, S., Wark, S., Street, G., Farley, J.W., and Brumley, W. C. (2002) Investigation of CE/LIF as a tool in the characterization of sewage effluent for fluorescent acidics: Determination of salicylic acid", Electrophoresis 23 (14), 2327-2332.

24. Jung, M. and Brumley, W. C. (1995) Trace analysis of fluorescein-derivatized phenoxy acid herbicides by micellar electrokinetic chromatography with laser-induced fluorescence detection. Chromatogr. A 717, 299-308.

25. El Rassi, Z., Mechref, Y., Postlewait, J., and Ostrander, G. K. (1997) Capillary Electrophoresis of

26. Carboxylated carbohydrates. III. Selective Precolumn Derivatization of Glycosaminoglycan Disaccharides with 7-Aminonaphthalene-1, 3-Disulfonic Acid Fluorescing Tag. Anal. Biochem. 244, 283-290.

27. Kok S. J., Kristenson, E. M., Gooijer, C., Velthorst, N. H., and Brinkman, U. A. Th. (1977)

28. Mikkelsen SR, Cortón E. Capillary electrophoresis in bioanalytical chemistry application's, John NJ, USA. 2004.

29. Skoog, D.A.; Holler, F.J.; Crouch, S.R "Principles of Instrumental Analysis" 6th ed. Chapter 30 Thomson Brooks/Cole Publishing: Belmont, CA 2007.

30. Skoog, D.A.; Holler, F.J.; Nieman, T.A. "Principles of Instrumental Analysis, 5th ed." Saunders college publishing: Philadelphia, 1998.

31. Altria KD, Elder D. Overview of the status and applications of capillary electrophoresis to the sample.

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