# DEVELOPMENT AND VALIDATION OF ONLINE PRECONCENTRATION-CAPILLARY ELECTROPHORESIS METHODS FOR THE DETERMINATION OF SELECTED PHARMACEUTICAL COMPOUNDS IN AQUEOUS MATRICES

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**by**

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## LIST OF SYMBOLS

| | |
|---|---|
| °C | degree celsius |
| µg/mL | microgram per milliliter |
| µm | micrometer |
| cm | centimeter |
| i.d. | internal diameter |
| kV | kilovoltage |
| M | molarity |
| mAU | milli-absorbance unit |
| mBar | millibar |
| min | minutes |
| mL | milliliter |
| mM | milli molar |
| MΩ $cm^{-1}$ | Mega ohm per centimeter |
| ng/mL | nanogram per milliliter |
| nm | nanometer |
| s | seconds |

## LIST OF ABBREVIATIONS

| | |
|---|---|
| 5-dUrd | 5-Fluoro-2-Deoxyuridine |
| 5-FU | 5-Fluorouracil |
| AFMC | Analyte focusing micelle collapse |
| BGE | Background electrolyte |
| CE | Capillary electrophoresis |
| CZE | Capillary zone electrophoresis |
| DAD | Diode array detector |
| EKI | Electrokinetic injection |
| EKS | Electrokinetic supercharging |
| EOF | Electroosmotic flow |
| EPF | Electrophoretic flow |
| FASS | Field amplified sample stacking |
| FASI | Field amplified sample injection |
| FESI | Field enhanced sample injection |
| FSCE | Free Solution Capillary Electrophoresis |
| HDI | Hydrodynamic injection |
| LLE | Liquid-liquid extraction |
| MEEKC | Microemulsion electrokinetic chromatography |
| MEKC | Micellar electrokinetic chromatography |
| MSS | Micelle to solvent stacking |
| PAEKI | Pressure assisted electrokinetic supercharging |
| PST | N4-phthalylsulfathiazole |
| SA | Sulfanilamide |
| SALLE | Salting out liquid-liquid extraction |
| SCIZ | Sulfaclozine |
| SCP | Sulfachloropyridazine |
| SDD | Sulfadimidine |
| SDM | Sulfadimethoxine |
| SDM | Sulfadimethoxine |
| SDS | Sodium dodecyl sulphate |
| SDX | Sulfadioxine |

| | |
|---|---|
| SDZ | Sulfadiazine |
| SGN | Sulfaguanidine |
| SIM | Sequential injection mode |
| SIX | Sulfisoxazole |
| SM | 5-Sulfamerazine |
| SMI | Sulfamethizole |
| SMM | Sulfamonomethoxine |
| SMP | Sulfamethoxypyridazine |
| SMR | Sulfamerazine |
| SMT | Sulfameter |
| SMZ | Sulfamethaxazole |
| SMZ | Sulfamethazine |
| SPD | Sulfapyridine |
| SPE | Solid phase extraction |
| SPZ | Sulfaphenazole |
| SPZ | Sulphenazole |
| SQL | Sulfaquinaxoline |
| SQX | Sulfaquinoxaline |
| SST | Succinylsulfathiazole |
| SSZ | Sulfisoxazole |
| SAc | Sulfacetamide |
| STZ | Sulfathiazole |
| SXL | Sulfamoxole |
| t-ITP | Transient isotachopherosis |

## LIST OF APPENDICES

**PEMBANGUNAN DAN PENGESAHSAHIHAN TEKNIK PRAPEMEKATAN ATAS TALIAN-ELEKTROFORESIS RERAMBUT BAGI PENENTUAN SEBATIAN FARMASEUTIKAL TERPILIH DI DALAM SAMPEL AKUEUS**

## ABSTRAK

Dalam kajian ini, beberapa teknik prapemekatan dalam elektrolisis rerambut (CE) dibangunkan untuk meningkatkan kepekaan untuk pengesanan sebatian farmaseutikal dalam matriks akueus sebenar. Ini disebabkan oleh kekangan utama untuk membangunkan kaedah analisis yang mudah dan sensitif untuk kajian analisis sebatian farmaseutikal analit terpilih (antikanser, antibiotik dan ubat anti-radang bukan steroid) adalah sangat terhad. Tahap gangguan yang lebih tinggi dalam matriks yang kompleks (biologi, makanan dan air) dengan keupayaan pengesanan instrumen analisis yang rendah membawa kepada pembangunan teknik prapemekatan dalam talian. Teknik pertama disebut pengenalan mod suntikan analit secara berturutan digunakan untuk pemisahan dan penentuan agen antikanser 5-fluorourasil (5-FU) dan metabolitnya iaitu 5-fluoro-2-deoksiuridin (5-FdUrd) di dalam plasma manusia yang diperoleh daripada pesakit kanser. Teknik ini melibatkan pengenalan jumlah sampel yang lebih banyak secara hidrodinamik dan dituruti secara elektrokinetik secara berasingan. Di bawah keadaan eksperimen yang optimum, peningkatan kepekaan bertambah dalam nilai 614- dan 643- kali ganda dan 782- dan 803- kali ganda untuk 5-FU dan metabolitnya jika dibandingkan dengan pengenalan sampel yang minimum secara normal menggunakan mod hidrodinamik dan elektrokinetik secara asing. Kaedah ini mempunyai julat linear yang baik (1–1000 ng/mL) dengan keluk tentukuran yang dapat diterima ($r^2 \geq 0.993$), had pengesanan yang rendah (0.11–0.14 ng/ml) dan pengembalian yang memuaskan (97.4–99.7%) dengan kepersisan dalam

julat 4.6–9.3% (*n=6*). Hasil pengesahan analisis diterapkan pada sampel plasma manusia iaitu daripada pesakit kanser dan menunjukkan teknik ini sesuai untuk kajian klinikal. Teknik seterusnya ialah penyimpangan pH dinamik untuk pengesanan antibiotik sulfonamida dalam sampel susu dan yogurt. Teknik ini melibatkan rentetan pH yang berbeza antara larutan elektrolit latar belakang (BGE), sampel dan larutan penamat (larutan asid) menghasilkan bilangan ion analit bertambah dan pembentukan jalur sempit pekat antara zon. Kelinearan yang baik dalam julat 30–500 ng/mL diperoleh dengan nilai kolerasi penentuan ($r^2 \geq 0.9940$), had pengesanan yang rendah (4.1–6.5 ng/mL), dan pengembalian yang memuaskan (81.2–106.9%) dengan RSD (5.3–13.7%, *n=9*) diperoleh dalam keadaan optimum. Faktor peningkatan kepekaan diperoleh adalah 231 kali ganda untuk sulfametoksazol, sulfametazin, sulfadiazin, sulfamonometoksin, sulfadimetoksin dan sulfasetamida untuk dua jenis sampel iaitu susu dan yogurt. Kaedah terakhir yang dicadangkan dalam kajian ialah pengekstrakan mikro cecair-cecair penyebaran digabungkan dengan isotakoforesis penyapuan-fana yang telah dibangunkan untuk analisis tiga ubat anti-radang bukan steroid iaitu ketoprofen, naproksen, dan natrium diklofenak dalam sampel air persekitaran. Teknik ini berdasarkan sistem larutan elektrolit secara putus yang disebut sebagai sistem penumpuan dalam keadaan tetap yang bergantung kepada mobiliti larutan tersendiri dengan konduksi yang berbeza melibatkan BGE misel, elektrolit mula dan penamat serta larutan sampel tanpa misel. Analit yang disapu akan terkumpul dalam jalur sempit dan seterusnya meningkatkan kepekaan pengesanan sebanyak 666 kali ganda. Pada keadaan yang optimum, kelinearan yang baik diperoleh dalam julat 0.1–500 ng / mL ($r^2 \geq 0.998$), had pengesanan yang rendah (0.01–0.07 ng / mL), dan pengembalian (99.6–101.9%) dengan RSD (1.4–8.6%, *n=9*) telah dicapai untuk analit yang dikaji. Teknik yang digunakan menunjukkan faktor peningkatan kepekaan yang baik, langkah

rawatan sampel juga menjadi lebih mudah dengan julat linear yang memuaskan, pengembalian dan kepersisan yang tinggi serta kos yang lebih efektif dengan penggunaan sampel dan pelarut secara minimum dapat digunakan untuk penentuan analit secara serentak dalam masa yang singkat.

**DEVELOPMENT AND VALIDATION OF ONLINE PRECONCENTRATION-CAPILLARY ELECTROPHORESIS TECHNIQUES FOR THE DETERMINATION OF SELECTED PHARMACEUTICAL COMPOUNDS IN AQUEOUS MATRICES**

## ABSTRACT

In this study, several preconcentration strategies in capillary electrophoresis (CE) were developed to improve the sensitivity of detection of pharmaceutical compound in real aqueous matrices. This is due to the core challenge to develop simple and sensitive analytical method for trace analysis study of selected analytes (anticancer, antibiotic and non-steroidal anti-inflammatory drugs) are very limited. Higher interference level from complex matrix (biological, food and water) with analytical instrument poor detection capability directed to development of online preconcentration technique. The first technique termed sequential injection mode stacking was developed for the separation and determination of anticancer agent 5-fluorouracil (5-FU) and its metabolite namely, 5-fluoro-2-deoxyuridine (5-FdUrd) in human plasma obtained from cancer patients. In this technique, large sample volume was introduced through electrokinetic injection followed by hydrodynamic injection. Under the optimized experimental conditions, 614- and 643- fold and 782- and 803-fold sensitivity improvement was obtained for both 5-FU and its metabolite when compared with normal hydrodynamic and electrokinetic injection alone, respectively. The method has good linearity (1–1000 ng/mL) with acceptable coefficient of determination ($r^2 \geq 0.993$), low limits of detection (0.11–0.14 ng/ml) and satisfactory analyte relative recovery (97.4–99.7%) with relative standard deviations of 4.6–9.3% ($n=6$). Validation results as well as the application to analysis of human plasma

samples to cancer patients demonstrate the applicability of the proposed method to clinical studies. The next preconcentration technique known as dynamic pH barrage junction was proposed for the analysis and quantification of sulfonamide antibiotics in milk and yoghurt samples. The approach in dynamic pH barrage junction is based on the different pH barrage or zone between background electrolyte (BGE), sample and terminating plug (acid plug) expand the analyte ionization state and resulted in concentrated narrow band between zones. Good linearity in the range of 30–500 ng/mL ($r^2 \geq 0.9940$), low limits of detection (4.1–6.5 ng/mL), and acceptable analyte recovery (81.2–106.9%) with RSDs of 5.3–13.7% ($n=9$) were obtained under the optimized conditions. Sensitivity enhancement factors of up to 231 in the analysis of sulfamethoxazole, sulfamethazine, sulfadiazine, sulfamonomethoxine, sulfadimethoxine and sulfacetamide in both milk and yogurt samples. Finally, in the last methodology proposed in this study, dispersive liquid-liquid microextraction combined with sweeping-transient isotachophoresis was established for the trace level analysis of three non-steroidal anti-inflammatory drugs, namely ketoprofen, naproxen, and diclofenac, in environmental water samples. The stacking technique is performed based on the discontinuous electrolyte solution system referred as a steady state stacking system with its own mobilities at different conductivity that includes micellar BGE, leading and terminating electrolyte with sample solution void of micelle. The analytes were swept and stacked in narrow band and consequently enhanced the detection sensitivity factor of 666-folds. Under optimum parameters, good linearity in the range of 0.1–500 ng/mL ($r^2 \geq 0.998$), low limits of detection (0.01–0.07 ng/mL), and acceptable analyte recovery (99.6–101.9%) with RSDs of 1.4-8.6% ($n=9$) were achieved for the studied analytes. The developed techniques demonstrated good sensitivity enhancement factors, greatly simplified sample pretreatment procedure

with satisfactory linearity, high recovery and precision as well as cost effectiveness due to small sample and solvent consumption for rapid simultaneous determination of targeted analytes.

# CHAPTER 1

# INTRODUCTION

## 1.1 Capillary Electrophoresis

Capillary electrophoresis (CE) can be described as high-efficiency separation method of sample ions in a narrow bore (25-100 μm) capillary tube that is filled with a background electrolyte (BGE) or a buffer solution. The capillary is first filled with the required buffer solution. The capillary ends are then dipped into reservoirs/ vials at the inlet and outlet BGE solution containing high-voltage electrodes. The ion will move toward the appropriate electrode and pass through the detector (Figure 1.1). The solution ion 's migration rate or mobility is largely determined by its size and number of ionic charges (Landers, 1998; Hirokawa, 2018).

In short, a smaller ion will move faster than a larger ion with the same number of charges. Similarly, an ion with two charges will move faster than an ion with only one charge and similar size. Therefore, when the separation of hypothetical mixture of ions having different charges and sizes; highly charged ions will migrate and detected first. The electroosmotic flow (EOF) effectively pumps solute ions along the capillary toward the detector to the charge on the capillary, the buffer viscosity, and dielectric constant of the buffer. The smaller anions fight more strongly against the EOF and are therefore detected later than anions with a lower mobility. Multiply charged anions will migrate more strongly against the EOF and will be detected later (Glynn *et al.*, 1998; Landers, 1998). Different from the electrophoretic separation in CE, the separation in high performance liquid chromatography (HPLC) and gas chromatography (GC) is based on chromatography technique, whereby the compounds will interact directly with the mobile

phase and stationary phase in the system. General comparison between CE, HPLC and GC are shown in Table 1.1.

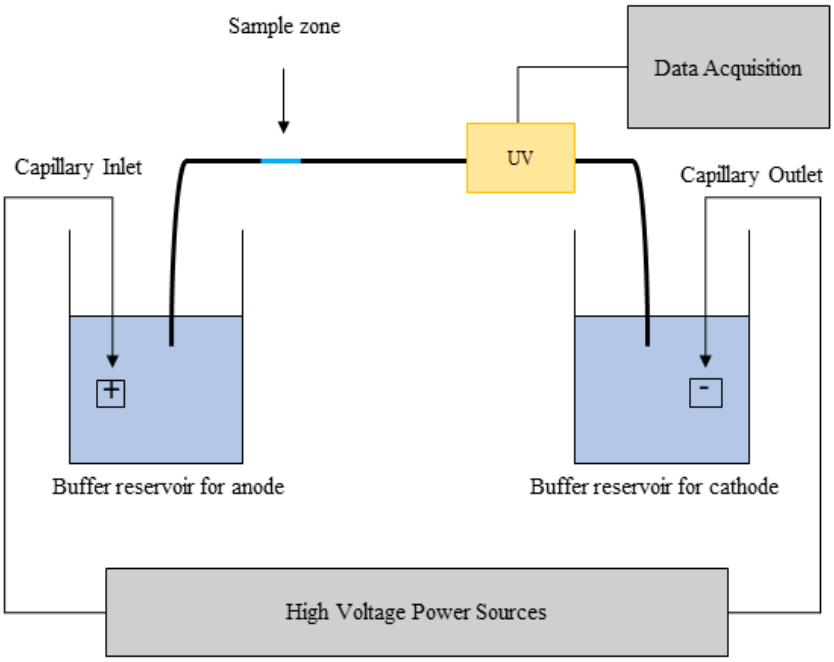


Figure 1.1 Capillary electrophoresis system (Glynn *et al.*, 1998)

The pH of BGE is obviously identified as the key operating parameter affecting the separation of ionic species, since it directs both the solute charged state and the level of EOF. The overall migration time of a solute is therefore related to both the mobility of the solute and EOF. The separation of ions is the simplest form of CE and is often termed Free Solution Capillary Electrophoresis (FSCE).

The separation and quantitation of chiral samples are an important area in many industries. Capillaries are filled with HPLC packing material, and the application of a voltage results in the EOF pumping the mobile phase through the capillary. Several separation modes applied in CE known as capillary zone electrophoresis (CZE), micellar

electrokinetic chromatography (MEKC), capillary gel electrophoresis (CGE) and capillary isotachophoresis (CITP) where the analytes are separated based on charge-to-mass ratios, chromatographic partition of analytes between micelles and the BGE, capillary analytes' isoelectric points, based on moving boundaries. These anionic micelles migrate against the EOF and can chromatographically interact with ionic and neutral solutes improved the separation efficiency. Two modes as for introduction of sample solution into the capillary are known as hydrodynamic (HDI) and electrokinetic (EKI) whereby samples were introduced by pressure and voltage, respectively.

CE has become a choice of separation technique because of its simple operation, cost-effective, high resolution, relatively short analysis time with the minimum consumption of chemicals and samples. However, the sensitivity of CE, especially with diode array detection, CE-DAD is limited due to the small injection volume and narrow optical path. The effective path length only covered about ~ 60% from the stated i.d. and yield a short online detection from the detector. Part of the capillary (lumen) in the window occupies only slight part (Figure 1.2) and causing the absorbance signal obtained correspondingly very small in path length. Noise levels are correspondingly small and are usually measured in micro-absorbance units. The maximum absorbance of typical CE detectors is 0.2 AU (Altria, 1996; Landers, 2007). The online preconcentration approaches are noted for their ability to increase the sensitivity in CE. They can be performed conveniently, by adjusting the electrolyte solution composition, the sample matrix composition, and the sample injection procedure (Gao *et al.*, 2019).

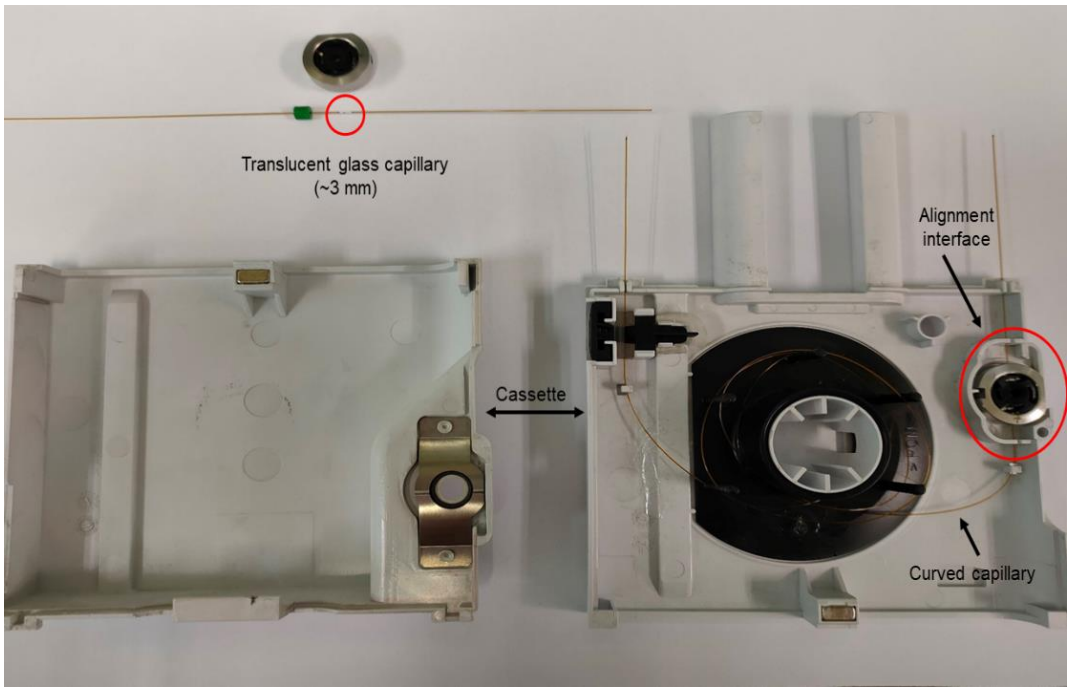


Figure 1.2 Capillary cassette and alignment interface of CE.

Table 1.1 Comparison on CE, HPLC and GC as analytical instrument.

| Technique | Capillary Electrophoresis (CE) | High Performance Liquid Chromatography (HPLC) | Gas Chromatography (GC) |
|---|---|---|---|
| Basic principle | Electrophoresis is the separation movement of ions based on mass to charge ratio in an electric field and flows through the capillary electro-osmotically or plug-flow rather than laminar (Altria, 1996; Landers, 1998) | Separation of chemical constituents mainly distributes by utilizing organic solvents as mobile phase and a packed column as stationary phase under high pressure based on the compound polarity and absorb -desorb process on column (Bélanger, Jocelyn Paré & Sigouin, 1997). | The separation work on the principle where the small and volatile compound in mixture injected into capillary column at high temperature separated into individual compounds carried by heated gas act as carrier (Khimii, 1977). |
| Cost and complexity | Maintenance is less expensive due to economical price of fused silica capillary (column). The analysis is faster and provides better resolution while using the least amount buffer solution and provides greater resolution, and is more efficient. System is largely reliant on the pH of the buffer solution, voltage and temperature for analyte separation (Michalke, 1999). | Costly due to large quantities of expensive organic solvents required for mobile phase and packed column.<br><br>Since various components have different properties, columns, and mobile phases are not suited for all types of substances and samples, it is difficult to design alternative procedures, which has led to the creation of a variety of offline preconcentration steps. | Due to the high cost of inert gas per tank, open tubular column, and derivatizing agent, this automated instrument is costly.<br><br>Difficult to establish a method since certain compounds must be chemically derivatized to become volatile, less polar, and thermally stable (Vrinat et al., 1988; Yilmaz & Ciltas, 2015; Wolecki et al., 2019; Giménez-Campillo et al., 2021). |

Table 1.1 Cont.

| Technique | Capillary Electrophoresis (CE) | High Performance Liquid Chromatography (HPLC) | Gas Chromatography (GC) |
|---|---|---|---|
| Speed, Efficiency and Accuracy | CE drive by applying voltage and background electrolyte/ buffer as mobile phase in minimum amount less than 2 mL per vial and small amount of sample with total analysis 5- 30 minutes.<br><br>Good injection practise; HDI mode by pressure is highly reproducible while electrokinetic by voltage is selective towards higher migration ions of target compounds resulted in precise and accurate detection (Krivácsy et al., 1999; Breadmore, 2009; Botello et al., 2013). Integration of compatible buffer and sample plug system, able to practise online preconcentration fully automated without affecting resolution (Bhargav KS, 2014; Voeten et al., 2018). | As HPLC employs a pump instead of gravity to drive a liquid solvent through a solid adsorbent material, various chemical components could well be separated quickly and efficiently. With high resolution, the total analysis time may be completed in around 3 to 30 minutes (Velikinac et al., 2004; Carabias-Martínez et al., 2006; Bizzotto et al., 2013).<br><br>Mainly automated as a one unit with a precise built-in solvent pump and a pressure unit, the analysis highly reproducible and accurate with injection volume from 5-50 µL (Kawasaki, Niikura & Kobayashi, 1990; Bélanger, Jocelyn Paré & Sigouin, 1997; Kočevar et al., 2008; Zhou et al., 2021). | Most volatile or derivatized non-volatile compound can be separated efficiently with heated gas as carrier. Fully automated with sample injection and total analysis mostly less than 30 minutes (Al-bukhaiti et al., 2017).<br><br>Include split injection for highly concentrated sample and also splitless injection mode for detection of trace compounds with high reproducibility even with a less sensitive detector. |

Table 1.1 Cont.

| Technique | Capillary Electrophoresis (CE) | High Performance Liquid Chromatography (HPLC) | Gas Chromatography (GC) |
|---|---|---|---|
| Sensitivity and Resolution | Detection occurs as resolved components move past a detector, typically UV/ diode array detector (DAD)/ capacitively-coupled contactless conductivity detector ($C^4D$)/ mass spectrometry (MS) and simple operating system which is vast and flexible for a wide spectrum of compounds, both ionic and non-ionic.<br><br>Limitation of the detection sensitivity due to optical detectors with short pathlength (Mikkers, Everaerts & Verheggen, 1979) Poor detection with optical detectors led to development of online preconcentration technique with enhanced separation efficiency even in highly complex matrix without prior sample dilution or extractions (Castro-puyana, Crego & Marina, 2008; Zhang, Zhang & Liu, 2013). | Detection occurs as resolved components move past a detector, typically UV/ DAD/ laser induced fluorescence (LIF). Adaptable and extremely accurate when it comes to qualify and quantify chemical components. With many optimized parameters involved, the precision of HPLC being automated is highly reproducible with less of manual handling.<br><br>HPLC does have trace level of detection sensitivity for certain compounds, and some cannot be detected due to the highly complex sample matrix. It is also not suitable for non- chromophore compounds for optical and different type of detectors (Freimüller & Altorfer, 2002; Sajid *et al.*, 2013). | Detection occurs as resolved components move past a detector, typically flame ionization detector (FID)/ electron capture detector (ECD) and mass spectrometry (MS).<br><br>Different type of detector have its own general purpose analyte detection at specified concentration limit which is highly sensitive and selective (Poole, 2016; Shimadzu, 2020). |

## 1.2 Sensitivity Enhancement in CE

Since its inception in the late 1980s, capillary electrophoresis (CE) has shown to be an advantageous tool for analysing a wide range of compounds. Its features include high efficiency, high resolving power, fast, cheap, full automation, and a range of injection-based preconcentration techniques. However, the main drawbacks from the conventional CE-UV escalate from two main sources; low sample injection volume and short optical length causing poor concentration sensitivity (Mikkers, Everaerts & Verheggen, 1979; Landers, 1998). Preconcentration is a process that convert the proportion of a desired trace element from the original into a new matrix appropriate for analytical trace analysis with improved detection sensitivity. In general, these preconcentration strategies can be classified into two categories; offline and online which have been applied in single or combination that known as sample extraction/ stacking method. Offline preconcentration methods that is normally applied for sample treatment before analytical detection were necessary to enhance CE detection sensitivity before online preconcentration was introduced. Detection sensitivity or signal detection in CE can be improved by off-line preconcentration strategies such as development of sample preparation for sample enrichment and isolation. Several sample preparation strategies such as solid phase extraction, liquid-liquid extraction and microextraction techniques have been explored for the CE applications. However, some of the procedures require time-consuming multi-step sample preparation, some are relatively expensive, and some require the use of a tremendous amount of organic solvents, and as a result, most of the analysis time is spent on the sample preparation steps (Bidari *et al.*, 2011). Several approaches have been employed to increase the path length for better detection and increased signal capability. One of the approaches is by using special design or fabrication of bubble capillary with increased path length (by

three-fold) where 50-µm i.d. capillary has a window that is 150 µm diameter in size (Palmer, Munro & Landers, 1999; Kawamura, 2011). Another strategy is through modifying or manipulating injection mode as online preconcentration technique (on-column) detection to obtained maximum capacity of signal to noise ratio or method detection limits. The on-column application enhanced the detection sensitivity by utilizing injection mode with longer injection of sample solution.

The technique depends on the analyte electrophoretic mobility after a lengthy plug of sample has been injected via a change in electric field (Osbourn, Weiss & Lunte, 2000; Breadmore, 2009). This approach is a well-known subject area in the field of electrophoresis and it is expected to remain a weight in pursuit to develop sensitive, universal systems for a variety of applications. Over the years, important points have been addressed in efforts to cope with complex matrix effects, as well as to minimize and simplify with more robust methods, especially for execution on portable devices. The main objective of fully automated portable device is to limit and reduce the obligation for highly complex manual sample handling. Published materials related to stacking or preconcentration techniques in CE from 1990 - 2020 (Figure 1.3) clearly shows that the field is growing rapidly where in 1996, 24 documents were published compared to about 2-9 from 1990-1995 (Figure 1.3). Comprehensive literature review on online preconcentration techniques in CE is discussed widely in this chapter.

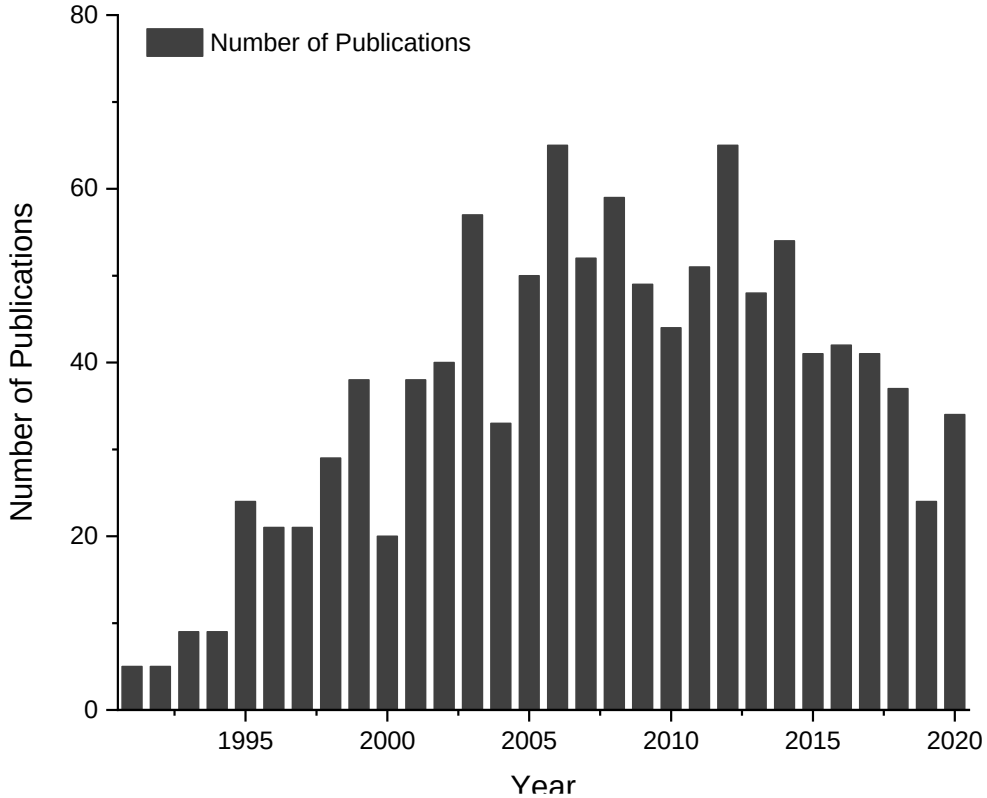


Figure 1.3 Growth of the publication devoted to stacking techniques in CE since 1990. Data attained from Scopus web of knowledge via stacking as search keyword (accessed on June 2021).

## 1.3 General Problem Statement

As discussed above, CE has been used as separation technique because of its simple operation, cost-effectiveness, high resolving power, relatively short analysis time, and minimum consumption of chemicals and samples. Despite the advances in CE detection and instrumentation, the small volumes of sample injection remain a limitation to attain adequate LODs for measuring minute concentrations of targeted analytes especially in complex matrices. The scope of this work based on the modification of the separation condition with new column coating by using surfactant or organic solvent as additives. The construction of new separation setup to facilitate better separation with several developments of preconcentration techniques was done on determination of selected pharmaceutical compound. The modification involved

electrolyte systems that affect the net velocity of target analyte in ionic form and altered the migration velocity capable in improving the detection capability using CE with optical detector. The implementation of several online preconcentration technique optimized and adopted under optimum condition applied for qualification and quantitation of target analyte with varied physico-chemical characteristic in aqueous sample matrix in trace level. In reviewing the existing literature, the development of online preconcentration strategies towards targeted analytes in this study (5-FU and its metabolite, SAs and NSAIDs) is very limited. Thus, it is important to establish online preconcentration strategies towards these targeted analytes, especially in the analysis of traces organic compounds to reach their maximum residue limits in specific matrices. Online preconcentration or stacking strategy was performed by adjusting the electrolyte solution composition, the sample matrix composition, and the sample injection step. The developed methods could serve as excellent alternative methods for practical applications with rapid analysis time and cost-effective analytical methodology in the future. The specific problem statements for each project carried out in this thesis are:

- The development of sensitive and reliable analytical methods for measurement of the concentration of 5-FU and its metabolites in plasma samples are needed to permit the design of more effective schedules of 5-FU administration in cancer treatment routines (Chapter 2).
- The main challenge to develop a sensitive and accurate analytical method for measurement of SAs in milk at the maximum residue limits of 100 µg/kg (Chapter 3) because isolated sulfonamides comprise both an acid and a base residue (amphoteric) that display a range of characteristics making separation more difficult especially from sample matrix with higher level of interferences.

- The main challenge to develop sensitive analytical method for detection of NSAIDS in environmental water samples for trace analysis at maximum residue limits of 0.35-28.0 ng/mL (Chapter 4) due to their various types of physico-chemical properties and occurrence in environmental water in ultra-trace level making the separation and detection hard to attain without preconcentration step.

## 1.4 General Objectives

From the above discussion, the development of online preconcentration strategy in CE is very important for the measurement of targeted analytes at minute concentration levels. Therefore, the general aim of this research study is to develop and establish online preconcentration techniques in CE combined with diode array detector as new alternative instrumental analytical methods for the analysis of 5-FU and its metabolite, SAs and NSAIDs compounds. The findings will serve as fundamental understanding towards the development of advance online preconcentration technique in the future. The specific aims of the project are to:

- To develop, optimize and validate sequential injection mode in micellar electrokinetic chromatography method for quantification of anticancer agent, 5-fluorouracil and its metabolite in human plasma (Chapter 2).
- To develop, optimize and validate dynamic pH junction for quantification of sulfonamides antibiotic in dairy product (Chapter 3).
- To develop, optimize and validate transient isotachophoresis and sweeping technique in determination of non-steroidal anti-inflammatory drugs in environmental water samples (Chapter 4).

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Online Preconcentration Techniques in Capillary Electrophoresis

Several approaches for preconcentrating samples and increasing the amount of sample that can be placed onto the capillary without affecting the separation efficiency have been developed. As an alternative to the conservative methods that applied offline preconcentration or without any preconcentration technique, online preconcentration can concentrate analytes with higher value of sensitivity enhancement factor (SEF) up to 200 000-folds and higher even with complex sample matrices (Hempel, 2000; Kitagawa & Otsuka, 2014). These techniques also termed as stacking methods due to the higher sample solution being stacked into a narrow zone or band in the capillary that results in higher response by detector. Several online stacking techniques have been developed such as field amplified sample stacking (FASS), field enhanced sample injection (FASI) or field enhanced sample injection (FESI), large volume sample stacking (LVSS), transient isotachophoresis (t-ITP), electrokinetic supercharging (EKS), counter flow gradient electro-focusing (CFGE) or pressure assisted electrokinetic injection (PAEKI), analyte focusing micelle collapse (AFMC), microemulsion electrokinetic chromatography (MEEKC), sweeping-micellar electrokinetic chromatography (sweeping-MEKC), micelle to solvent stacking (MSS) and dynamic pH junction. These above-mentioned techniques have been used as single and sometimes in combination of several techniques to overcome the short path length issue in CE online detection, and consequently enhanced the sensitivity and selectivity of the developed method in CE.

### 2.1.1 Field Amplified Sample Stacking and Field Amplified Sample Injection

The simplest stacking technique that is applicable in CE is known as field amplified sample stacking (FASS) and field amplified sample injection or field enhanced sample injection (FASI/FESI) using HDI and EKI, respectively. FASS and FASI/FESI are performed based on the low sample conductivity as compared to BGE.

The sensitivity enhancement is determined by the ratio of ion velocities in the sample zone and BGE zone. The signal sensitivity can be improved in 10–20 times compared to normal HDI with same BGE conductivity. Unfortunately, FASS technique is limited to the diluted sample solution and the sample plug capacity limited to only 3-5% of capillary volume due to sample matrix elimination step that is unable to performed in HDI mode, resulted in band broadening (Félez, Molet & Núñez, 2015). Despite the limitation FASS has been implemented in several recent routine analysis such as sulfonamide antibiotics (Alshana, Göğer & Ertaş, 2013; Liu *et al.*, 2017), psychiatric drugs (Dziomba, Biernacki, *et al.*, 2014), organic acid (Wei *et al.*, 2018) and alkaloids (Chu *et al.*, 2019). The band broadening effect can be eliminated with some modification on sample injection time with suitable capillary length for optimal separation capillary length after preconcentration without forfeiting the detection quality.

In FASI, the ion moved based on its EOF and electrophoretic mobility and susceptible to improve the sensitivity up to 1000-folds (Deng et al., 2014). The limitation of this technique is similar to FASS, whereby FASI only favours ions with higher electrophoretic mobility and the total ions injected into capillary depend on the conductivity of sample matrix. The samples are prepared in a low conductivity solution and electrokinetically injected into a high conductivity BGE where the ions stacked between two boundaries with different conductivities (Cao et al., 2016; Theurillat et

al., 2016). Mechanism of FASI is shown in Figure 2.1; the resistivity differences in both solutions causing the electric field strength in the sample zone is higher than BGE (Figure 2.1A). The ions are stacked in a narrow zone at the interface due to ion movement is relative to the electric field strength (Mikkers, Everaerts & Verheggen, 1979; Osbourn, Weiss & Lunte, 2000). The extremely short water plug (Figure 2.1B) was injected hydrodynamically in 4-10 seconds to establish a distinct boundary between the sample and the BGE zone, resulting in a stronger electric field at the injection end of the capillary acting as a rapid throughway for the charged analytes and further separated by normal CZE mode (Figure 2.1C). The improvement can be seen in the reproducibility and the resolution of analyte separation LOD and SEF enhancement of separation analyte (Hou *et al.*, 2010; Gao *et al.*, 2019; Zayed & Belal, 2020; Zarad *et al.*, 2021).

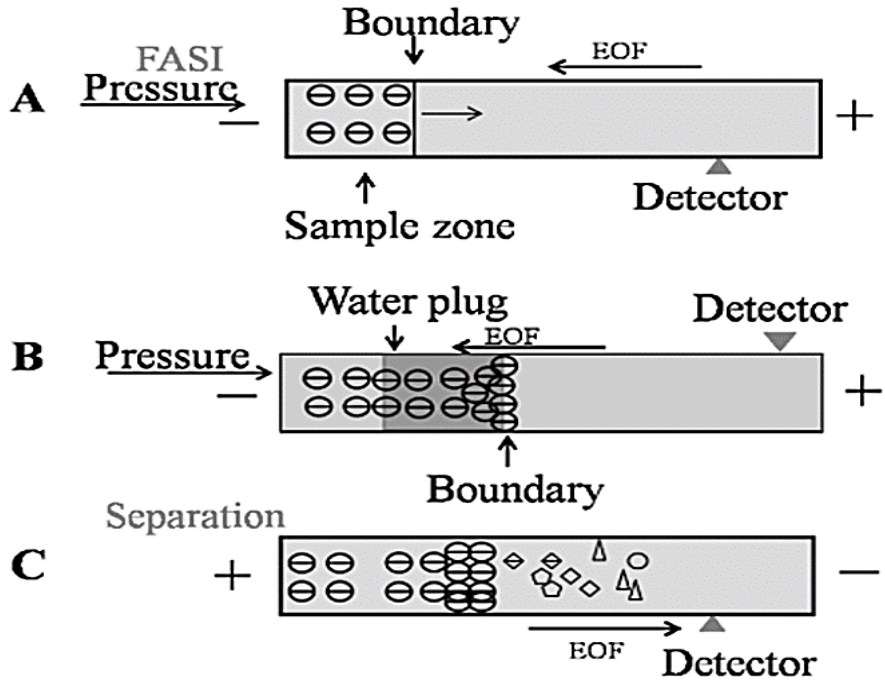


Figure 2.1 Stacking mechanism of FASI (Gao et al., 2019).

Applications on these both methods include the analysis of non-steroidal anti-inflammatory drugs, deferasirox, macrocyclic antibiotics and morphine in different type of sample matrices (Dawod *et al.*, 2008; Alshana, Göğer & Ertaş, 2013; Lin *et al.*, 2016; Hsieh *et al.*, 2017; Kowalski *et al.*, 2019; Zarad *et al.*, 2021), with sensitivity enhancement in range from 17 to 2000-folds were achieved. These two modes of injection also have been applied in combination as a hybrid sequential injection mode (HSIM) in the analysis of anti-fouling agent with enhanced signal intensity (peak height) of 30-folds. HSIM is a simple and capable approach for injection of different ion mobility in order to improve the sensitivity and reproducibility of target compounds (Kaewchuay et al., 2011). The prevalence of both FASI/ FESI and FASS research articles in pharmaceutical area were displayed in Figure 2.2 throughout the year from 1990-2021.

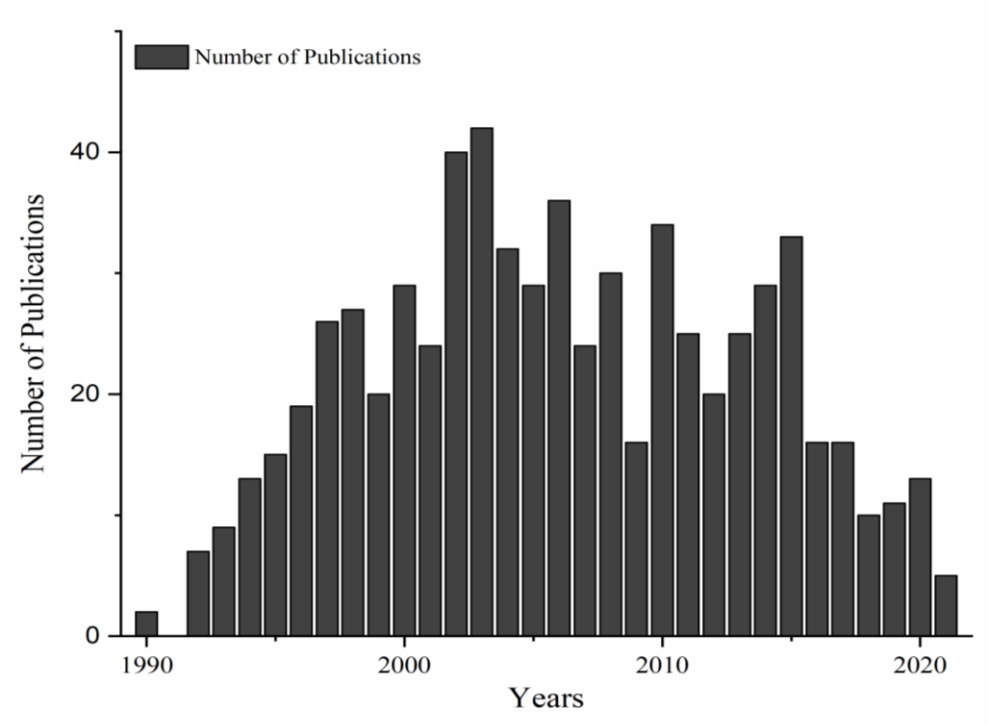


Figure 2.2 Occurrence of on-line concentration for electrophoresis as obtained from Scopus web using “FASS or FASI” in the topic. Number of papers for 1999 is up until June 2021.

### 2.1.2 Large Volume Sample Stacking

Large volume sample stacking (LVSS) is one of the stacking techniques that allowed injection of a large sample volume compressed into a narrow sample zone, contributing to sensitivity improvement in 50-100 folds. LVSS was first introduced by Chien and Burgi in 1992 (Chien & Burgi, 1992) for protein analysis. The sample were prepared by diluting it in aqueous solution, which is filled in 1/2 or 1/3 of the capillary. The sample was then introduced by gravitational force as in Figure 2.3a. Reverse polarity is applied with BGE at the detection end of the capillary and switched EOF to push the sample plug out of the capillary (Figure 2.3b). The analytes move toward the outlet end and stack at the boundary (Figure 2.3c) in a narrow band between sample plug and BGE zone and further separated by capillary zone electrophoresis as in Figure 2.3d (Chien & Burgi, 1992). By using LVSS, large volumes of sample can be introduced into the capillary which may then be concentrated online without shifting the peak shape.

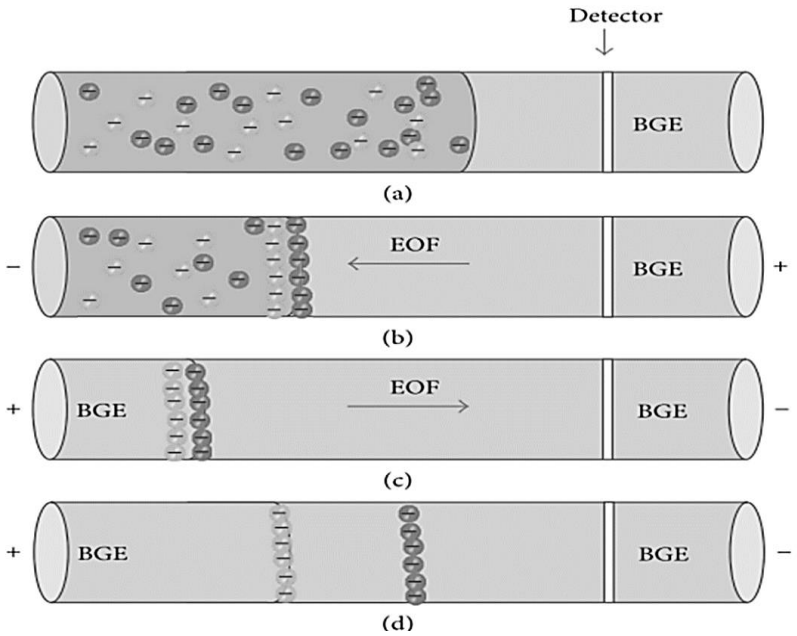


Figure 2.3 Mechanism of LVSS (Islas et al., 2018).

LVSS has been successfully applied in many applications including the analysis of phenols (Memon et al., 2019) antibiotics (Injac, Karljikovic-Rajic & Strukelj, 2008; Islas et al., 2018), naphthalenes and benxesulfonates (Cugat, Borrull & Calull, 2001), drugs (Yang et al., 2007) and preservatives (Cheng et al., 2012) with 5- to 118-fold enhancements were reported for the developed LVSS methods.

### 2.1.3 Transient Isotachophoresis

Isotachophoresis (ITP) is a straightforward electrophoretic separation method that separates charged components in an electric field due to differences in their electrophoretic mobilities. ITP is a stable and viable preconcentration technique which could concentrate a trace of a component in a high concentration of matrix ions based on the mobility of discontinuous electrolyte solution (Simpson, Quirino & Terabe, 2008; Grochocki, Markuszewski & Quirino, 2018; Hirokawa, 2018). Transient isotachophoresis (t-ITP) is the preconcentration term that applied in CE to describe the arrangement of a discontinuous electrolyte system involved two buffer systems namely leading electrolyte (LE) being introduced first followed by sample solution (S) and a terminating electrolyte (TE) (Figure 2.4a). These two buffers amplified the concentration of initial sample (S) in the migrating zone of the analyte at the ITP steady state (Busnel *et al.*, 2006). As soon as the analyte diffused in BGE, the analyte starts to drift away from its zone with new electric field (Figure 2.4b). The analytes (S) either accelerates or decelerates until it migrates back to its zone and the steady state is re-established (Figure 2.4c). When an electric field is applied the LE moves quickly (higher mobility) followed by S and TE in mobilities order (Okamoto & Hirokawa, 2003; Hirokawa, 2018).

Ions with mobility in between LE and TE will adjust their concentration in order to move at the same velocity as the LE bracketing zone. The electric field

strength is inversely proportional to the ion mobility in sample zone. A certain modification has been made to change analyte mobility that become faster by addition of LE in the sample solution to attained same t-ITP result with suitable LE/ TE and BGE system. Improved sensitivity can reach up to 10–1000 fold by using single or multiple ITP and the stacked sample zone can be coupled to the CZE for further separation and quantification using a single or separate capillary (Mai, Oukacine & Taverna, 2016).

Recently, a variation of ITP technique termed electrokinetic supercharging (EKS) has been developed. EKS is a powerful preconcentration technique where FASI and t-ITP used in combination for the analysis of diluted samples. It was first introduced by Hirokawa et al. in 2003 in the analysis of 18 cations, alkali metal ions and trivalent rare earth elements. The stacking mechanism of EKS starts with the introduction BGE, adequate amount of LE was filled and the sample injected by FASI. TE was filled subsequently and separation voltage was applied (Okamoto & Hirokawa, 2003). This procedure enabled the introduction of higher diluted sample volume without having peak broadening issues in separation. Among these pre concentration technique, EKS is one of the advantageous and practical approaches through combination of both FASI and t-ITP with higher detection as compared to single FASI and t-ITP. In EKS, the sample zone is stacked or being sandwiched between leading co-ion of LE and TE which is similar to t-ITP with higher amount of sample introduction that contributed to greater enhancement in sensitivity. Based on previously reported works, EKS has significantly improves the method sensitivity (based on peak area) by impacts resulting from 100 to 1800-fold, giving limits of detection as reduced as 100 ng / L (Dawod *et al.*, 2008; Tian *et al.*, 2013; Ye *et al.*, 2013; Karim *et al.*, 2016).

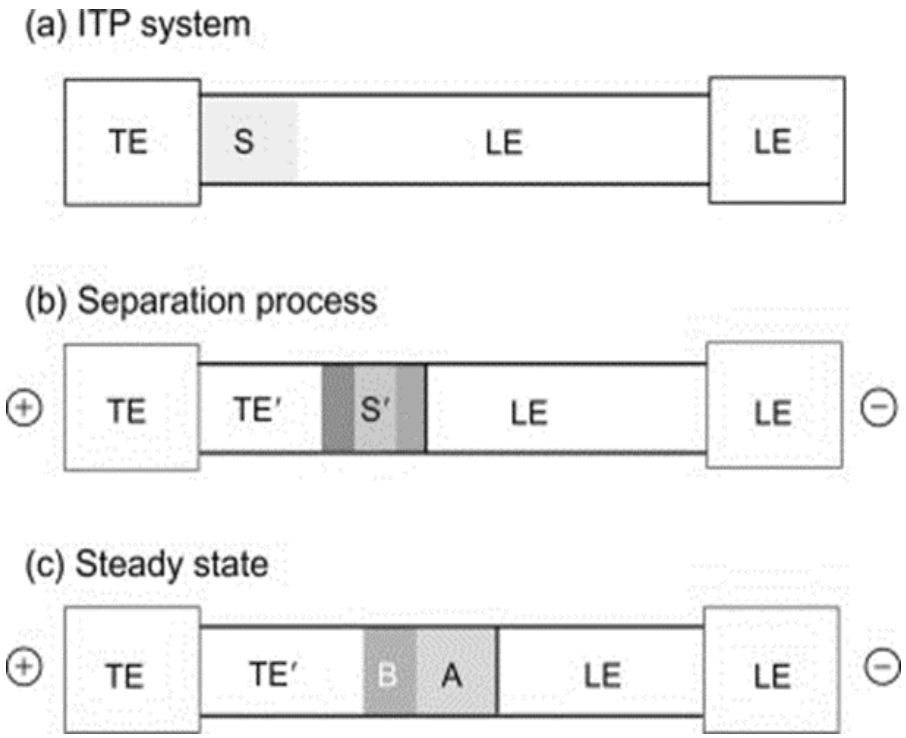


Figure 2.4 Mechanism of t-ITP in a capillary electrophoresis (Hirokawa, 2018).

### 2.1.4 Pressure Assisted Electrokinetic Supercharging

Pressure assisted electrokinetic injection (PAEKI), counter flow gradient electro-focusing or constant pressure assisted electrokinetic injection has been developed as the on-line sample enrichment technique by providing a powerful enhancement capability without sacrificing the separation efficiency. It is a relatively new method that combines FASI and tITP; PAEKI works based on these two stacking principles to achieve an unlimited enhancement power by applying an external pressure whilst balance the electroosmotic flow (EOF) in opposite for a potentially longer injection time (Fillet *et al.*, 1999; Meighan *et al.*, 2011; Zhang *et al.*, 2011; He, Xu & Ren, 2019). The injection order was led by BGE that also acts as leading electrolyte followed by long sample plug under reversed polarity aided by an external pressure.

The sample solution prepared in high resistivity and BGE solution in low resistivity concentration solution or vice versa so the analyte capable to stack in between the boundaries (Simpson, Quirino & Terabe, 2008). It was strongly recommended to suppress the EOF by applying external pressure or capillary coating to prevent any velocity mismatch of EOF that could induce band broadening (Feng, Lian & Zhu, 2007; Zhang *et al.*, 2011; D'Ulivo & Feng, 2015). Figure 2.5 showed the schematic of PAEKI procedure. In PAEKI, three injection modes that were applied for anionic analyte of interest where in Figure 2.5A and B are known conventional injection modes of HDI and EKI, respectively, attempt to inject higher sample volume by increasing plug injection time. In HDI, the injected sample volume is limited by the capacity of the capillary because a part of the capillary has to be left free of sample solution for the later separation. While EKI applied to the anionic compound with negative polarity as displayed in Figure 2.5B with reversed EOF pushing the analyte causing the depletion or analyte loss at the inlet. Thus, it is difficult to introduce a large amount of analyte by HDI and EKI alone. While in PAEKI, an external and constant pressure was applied during sample injection at the capillary inlet; since anionic analyte migrated to anode (positive end), the positive pressure helped to counteract the EOF.

The pressure needs to be optimized in order to find the best pressure that can establish a stable force to stabilize the EOF for complete analyte focusing. External pressure applied against viable EOF resulted in dynamic balance that almost become stationary boundary in PAEKI. A stable counterbalance of the EOF during injection will cause analyte to accumulate at the boundary between BGE and sample zone. As soon as the balance was achieved and a stationary zone/ immobile zone at the inlet of the capillary where the analytes stacked in a narrow sample zone according to FASI's

principle as in Figure 2.5C. Injection sample can be done either using HDI, EKI or both with longer sample plug length. In PAEKI, the capillary is capable to save space for later separation even longer sample plug is introduced into capillary.

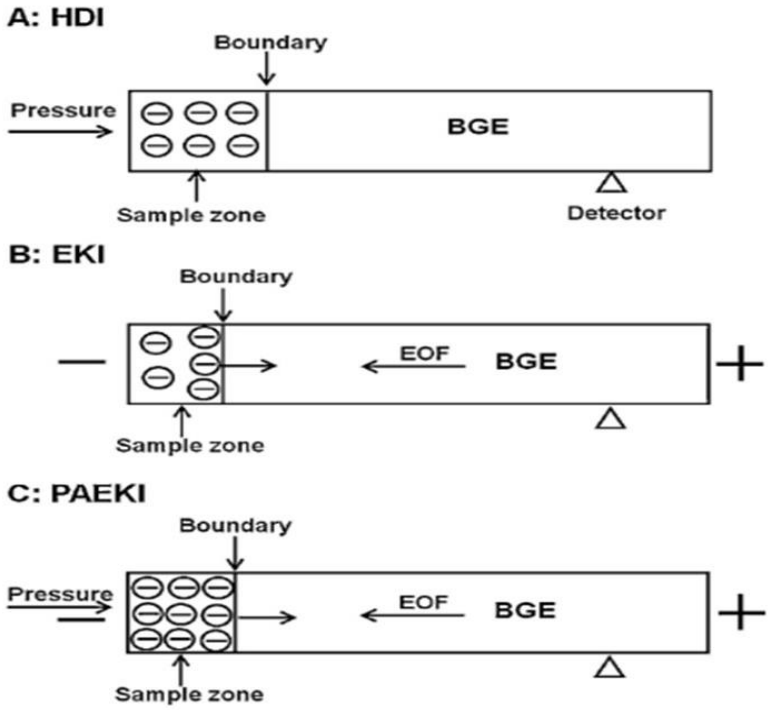


Figure 2.5 Pressure assisted electrokinetic supercharging mechanism (Xu *et al.*, 2014).

In PAEKI, the introduced sample plug was demonstrated to have a linear correlation with the ratio of resistivity (γ) of the sample matrix to the BGE in the capillary and the injection time (t) based on field amplification can be expressed by Eq. (1) (Feng & Zhu, 2006):

$$\boldsymbol{Ni(t)\gamma} = \mu\boldsymbol{epi\ Einj\ ACinj\ t} \qquad (\mathbf{1})$$

Where;

$\boldsymbol{\mu_{epi;}}$ analytes electrophoretic mobility in sample matrix/solution,

$E_{inj} = V_{inj}/ L$; strength of electric field equals to the injection voltage ($V_{inj}$) divided by the capillary length *(L*),

***A***; area of capillary cross section and,

$C_{inj;}$ analyte concentration in the sample solution.

The field amplification (preconcentration) factor of the injected amount of sample was determined by the diameter and length of capillary occupied by the sample zone itself, capillary length for separation efficiency and the EOF was balanced by external pressure in PAEKI for stable zone once voltage was applied during stacking (Feng & Zhu, 2006, 2008; Meighan *et al.*, 2011). The optimization of injection volume and voltage with suitable capillary length is a must to achieved a satisfactory separation efficiency and SEF.

The stacking mechanism and the migration under reverse polarity for anionic analyte. In PAEKI, the stacked sample will mount up in narrow zone even with unlimited sample injection. The limit of detection can reach far lower than EKS alone (Meighan *et al.*, 2011). Sample injection can be increased up to 2- 3 orders of magnitude using PAEKI technique and the amount is comparable to injection volume of high performance liquid chromatography (HPLC) (Feng, Lian & Zhu, 2007; Meighan *et al.*, 2011; Xu *et al.*, 2014). The complete focusing can be achieved when bulk velocity of the cation layer/ BGE layer is equal to the velocity of the pressure applied at the inlet with longer sample plug. The immobile boundary between analyte and cationic layer of BGE as the analyte moved toward anode due to negative charge and saving up space in capillary stacked in narrow band and being pushed by EOF during EKI sample injection. Meighan et al. reported PAEKI method and the enrichment achieved for NSAIDs separation analysis was in 50 000-folds under optimum parameters in positive polarity. This work applied higher injection voltage (-

14 kV) effectively opposed the EOF with no negative effect when applied higher injection volumes up to 45 minutes (Meighan *et al.*, 2011). Zhang et al. also reported PAEKI method for separation of ten haloacetic acid by utilizing short injection amount at -5kV and 50 mbar for external pressure at 3 minutes in negative polarity (-20 kV) for separation voltage. Under optimized condition the target analyte was successfully enriched up to 20 000-folds with LOD in 0.013-0.12 ng/ mL by eliminating sample matrices during stacking using EKI.

Unfortunately at a certain time, the amplified factor observed was not linear to the sample injection plug length since higher volume (> 5 minutes) at lower injection voltage (-5 kV) induced incomplete focusing that disrupting the balance of the stacked zone (Zhang *et al.*, 2011). This is due to partially damage/ total damage layer and the analyte band was not stacked properly due to pressure applied during sample injection is either too low or high affecting boundary stability causing analyte unable to stacked in narrow zone. The resolution will be deteriorated with increased amplification factor or longer sample plug since the balance were interrupted with joule heating. This will result poor resolution/ band broadening and affect the reproducibility of the separation (Zhang, Gavina & Feng, 2011).

### 2.1.5 Micelle-Based Stacking

Along with its high resolution and ability to discriminate ionic and neutral molecules concurrently, CE is a powerful method for detecting numerous compounds. Unfortunately for non-ionic at most pH values and hydrophobic analytes, the application of electrokinetic injection for sensitivity augmentation is impracticable because neutral analyte does not have electrophoretic mobility. Most of pharmaceutical substances are neutral from separations by the typical capillary zone electrophoresis which based on the variances of the analyte electrophoretic mobilities