การตรวจวัดยาลดความอ้วนในผลิตภัณฑ์เสริมอาหารเพื่อควบคุมน้ำหนัก โดยคะพิลลารีอิเล็กโทรฟอริซิส

นายณัฐพงษ์ ชมวะนา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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DETERMINATION OF ANTI-OBESITY DRUGS IN DIETARY SUPPLEMENTS FOR WEIGHT CONTROL BY CAPILLARY ELECTROPHORESIS

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A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title	Determination of anti-obesity drugs in dietary supplements for weight control by capillary electrophoresis
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ณัฐพงษ์ ชมวะนา : การตรวจวัดยาลดความอ้วนในผลิตภัณฑ์เสริมอาหารเพื่อควบคุมน้ำหนักโดยคะพิลลารีอิเล็กโทรฟอริซิส (DETERMINATION OF ANTI-OBESITY DRUGS IN DIETARY SUPPLEMENTS FOR WEIGHT CONTROL BY CAPILLARY ELECTROPHORESIS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร.ธรรมนูญ หนูจักร, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ.ดร. ณัฐชนัญ ลีพิพัฒน์ไพบูลย์, 96 หน้า.

งานวิจัยนี้เป็นการพัฒนาเทคนิคคะพิลลารีอิเล็กโทรฟอริซิส สำหรับการแยกในคราวเดียวกันและปริมาณวิเคราะห์ของยาลดความอ้วนที่ปนปลอมในผลิตภัณฑ์เสริมอาหารเพื่อควบคุมน้ำหนัก ได้แก่ เฟนเตอมีน, ไซบูทรามีน, อีฟีดรีน, ซูโดอีฟีดรีน และเฟนฟลูรามีน โดยภาวะที่เหมาะสมคือ ใช้ 100 mM ทริสฟอตเฟสบัฟเฟอร์ที่ pH 2.5 ประกอบด้วย 20% แอซิโทไนไทล์ ในคะพิลลารีขนาดเส้นผ่านศูนย์กลางขนาด 50 μm i.d. × 60.2 cm. (50 cm ถึงเครื่องตรวจวัด) ภายใต้ความต่างศักย์ไฟฟ้า 25 kV และอุณหภูมิที่ 25 องศาเซลเซียส พบว่าฐานพีกของสารแยกออกจากกันสมบูรณ์ด้วยค่าการแยกสารมากกว่า 2.0 ภายใน 10 นาที วิธีการนี้ได้ขีดจำกัดของการตรวจวัด (LOD) และขีดจำกัดของการวิเคราะห์เชิงปริมาณ (LOQ) อยู่ในช่วง 0.10-0.20 ppm และ 0.45-1.00 ppm ตามลำดับ ได้ค่าความเป็นเส้นตรงมากในช่วงความเข้มข้น LOQ-50 ppm ด้วยค่าสัมประสิทธิ์การกำหนด r^2 มากกว่า 0.999 ตามด้วยความเที่ยงภายในวันเดียวกันและระหว่างวันของเวลาในการวิเคราะห์และความเข้มข้นที่วัดได้อยู่ในเกณฑ์ที่ยอมรับได้ที่ค่าเบี่ยงเบนสัมพันธ์น้อยกว่า 1% สำหรับเวลาในการวิเคราะห์และน้อยกว่า 4.1% สำหรับความเข้มข้นที่วัดได้ ได้ความถูกต้องของปริมาณวิเคราะห์ที่ยอมรับได้ด้วยร้อยละการกลับคืนอยู่ในช่วง 96.3-104.9% ที่ความเข้มข้นของ LOD, 95.3-103.7% ที่ความเข้มข้น 5 ppm และ 96.1-104.7% ที่ความเข้มข้น 30 ppm จากการใช้วิธีการนี้สำหรับวิเคราะห์ตัวอย่างจริงชนิดแคปซูลที่สกัดด้วย 1:10 เอทานอลต่อน้ำ ตรวจพบไซบูทรามีนใน 4 ตัวอย่างจากทั้งหมด 8 ตัวอย่างในปริมาณมากถึง 2.7% w/w หรือ 6.4 มิลลิกรัมต่อแคปซูล นอกจากนี้พบว่า 20 mM แอมโมเนียมแอซิเตด ที่ pH 4 ที่ประกอบด้วย 30 ถึง 50% แอซิโทไนไทล์ หรือ 40 ถึง 70% เมทานอล ให้การแยกที่สมบูรณ์ของยาลดความอ้วนทั้ง 5 ชนิด ซึ่งอาจใช้เป็นบัฟเฟอร์ที่กลายเป็นไอในแมสสเปกโตรเมทรี และยังได้แมสสเปกตรัมของผลิตภัณฑ์ไอออนของยาลดความอ้วนทั้ง 5 ชนิด โดยใช้เทคนิคอิเล็กโตรสเปรย์-แทนเดมแมสสเปกโตรเมทรีที่มีเครื่องวิเคราะห์มวลเป็นควอดูโพล-ไทม์ออฟไฟต์

ภาควิชา เคมี ลายมือชื่อนิสิต .....

สาขาวิชา เคมี ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 2555 ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์ร่วม .....

5372402123 : MAJOR CHEMISTRY
KEYWORDS : CAPILLARY ZONE ELECTROPHORESIS / ANTI-OBESITY DRUGS / DIETARY SUPPLEMENTS

NATTHAPHONG CHOMVANA : DETERMINATION OF ANTI-OBESITY DRUGS IN DIETARY SUPPLEMENTS FOR WEIGHT CONTROL BY CAPILLARY ELECTROPHORESIS. ADVISOR : ASSOC. PROF. THUMNOON NHUJAK, Ph.D., CO-ADVISOR : ASST. PROF. NATCHANUN LEEPIPATPIBOON, DR.RER.NAT. 96 pp.

Capillary zone electrophoresis (CZE) was developed for simultaneous separation and quantitative analysis of five anti-obesity drugs adulterance in dietary supplements for weight control: phentermine, sibutramine, ephedrine, pseudoephedrine and fenfluramine. The suitable condition were obtained using a 100 mM tris-phosphate buffer at pH 2.5 containing 20% acetonitrile in a fused-silica capillary under applied voltage of 25 kV and temperature of 25 ^{o}C. Achieve base line resolution was obtained with R_s > 2.0 and analysis time within 10 min. This method allows the limits of detection (LOD) and limits of quantitation (LOQ) in ranges of 0.10-0.20 and 0.45-1.00 ppm, respectively. Highly linear relationship in concentrations levels of all analytes in a range of LOQ to 50 ppm was obtained with coefficient of determination r^2 > 0.999, along with acceptable intraday and interday precision in migration time (t_m) and the measured concentration shown by RSD of < 1.0% for t_m and < 4.1% for the measured concentration. Satisfactory accuracy was also obtained with recoveries of 96.3-104.9% at LOQ, 95.3-103.7% at 5 ppm and 96.1-104.7% at 30 ppm. Using this method for analysis of real samples extracted by 1:10 ethanol:water with proper dilution, sibutramine was found to be adulterated in four out of eight capsule samples of weight control, with the amount levels up to 2.7% w/w or 6.4 mg/capsule. In addition, a 20 mM ammonium acetate buffer at pH 4 containing 30 to 50% ACN or 40 to 70% MeOH was found to achieve CZE separation of five anti-obesity drugs, that may be used as a volatile buffer in mass spectrometry. Product ions mass spectra of five anti-obesity drugs were also obtained using electrospray ionization-tandem mass spectrometry with a quadrupole-time of fight mass analyzer.

Department : Chemistry Student's Signature .....
Field of Study : Chemistry Advisor's Signature .....
Academic Year : 2012 Co-advisor's Signature .....

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LIST OF ABBREVIATIONS

$\Delta\mu$	electrophoretic mobility difference
ΔT	temperature difference
A	degree of ionization
ε	dielectric constant
ε_0	permittivity of free space
H	viscosity
κ^{-1}	thickness of diffusion layer
μ	electrophoretic mobility
μ_{eo}	electroosmotic mobility
μ_{net}	total of electroosmotic mobility
μ_{eff}	apparent electroosmotic mobility
ν_{ep}	electrophoretic velocity
ν_{eo}	electroosmotic velocity
ν_{net}	total of velocity
σ	standard deviation of peak
ζ	zeta electric potential
A_{corr}	corrected peak area
C	concentration of sample
D	diffusion coefficient
E	electronic charge
E	electric field strength
f	dilution factor
f_T	constant (0.02-0.03 K^{-1})
L	the length of capillary to detector
L	total capillary length
N	efficiency or the number of theoretical plates
r^2	coefficient of determination
r_h	hydrodynamic radius of the ion
R_s	Resolution

t_m	migration time
T	temperature (K)
V	applied voltage
V_F	volume flow
W	peak width at base
Z	electronic charge
ACN	acetonirile
Asy	asymmetry peak
BGE	background electrolyte
CE	capillary electrophoresis
CEC	capillary electrochromatography
CGE	capillary gel electrophoresis
CIEF	capillary isoelectric focusing
CITP	capillary isotachophoresis
CZE	capillary zone electrophoresis
ESI	electrospray ionization
EMD	electromigration dispersion
EOF	electroosmotic flow
EtOH	ethanol
FDA	Food and Drug Administration
FLD	fluorescence detector
GC	gas chromatography
HPLC	high performance liquid chromatography
i.d.	internal diameter
IS	internal standard
LOD	limit of detection
LOQ	limit of quantitation
MEKC	micellar electrokinetic chromatography
MS	mass spectrometry
MeOH	methanol
MDL	method detection limit

MQL	method quatitation limit
Q-TOF	quadrupole-time of flight
SPE	solid phase extraction
S/N	signal to noise ratio
E	Ephedrine
F	Fenfluramine
P	Phentermine
PE	Pseudoephedrine
S	Sibutramine

CHAPTER I
INTRODUCTION

1.1 Problem Definition

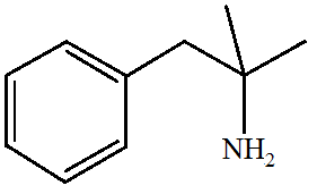
Up-to-date, a dietary supplement has been increasingly popular for weight-control or weight loss because it is simply used and provides effective management better than either dieting or exercise. However, some manufactures may adulterate anti-obesity drugs in dietary supplement for lowing cost and increasing the effective of lose weight without a control from clinical assessment [1]. Some drugs have been withdrawn or banned because they show the side effects to consumers who have taken overdoses of these drugs for long time. The consumers may risk a high chance of organ damage or even death. The Food and Drug Administration (FDA), United States (US) defines that dietary supplements cannot be advertised as diagnosing, preventing, treating or curing a disease, but they can be advertised to affect the function or structure of the body, and they containing ingredients, vitamin, mineral, herb or other botanical, amino acid, enzymes. The dietary supplement adulterated with these drugs is illegal under controlling of the Ministry of public Health. However, the illegal dietary supplements have been widely sold in the market, especially social networks that are difficult to control [2, 3]. Most of the anti-obesity drugs adulterated in dietary supplements are shown in Figure 1.1.

Phentermine (P) is a drug that affects the central nervous system around the hypothalamus region. It stimulates a release of norepinephrine and dopamine which suppresses hunger but it has a host of potential side effects such as headaches, insomnia, increased heart rate, high blood pressure and even risk of death. This substance has been registered as a weight loss drug suitable for short term use only (not exceeding 12 weeks). It should be consumed at a dosage of no more than 15-30 mg per day. It is classified as a grade 2 active substance, which means it cannot be produced, sold or imported without authorization from the Ministry of Public Health [6, 7].

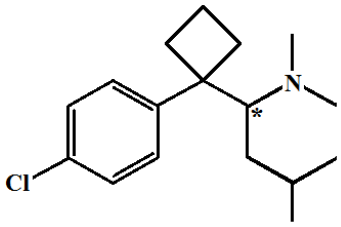
Sibutramine (S) is a serotonin and norepinephrine reuptake inhibitor for the management of obesity, which sibutramine exerts its weight-loss effect is likely due to reduced appetite, feelings of satiety, and increases the metabolic rate. The effective in stimulating the initial weight loss and maintenance of weight loss is well proven in short-term and long-term clinical trials of up to 2 years in duration. Although, sibutramine has an advantage, but may cause side effects that are harmful to consumers who have used for a long-time. Sibutramine might have opposite effects on peripheral and central sympathetic activity, an increase in blood pressure, insomnia, dry mouth and constipation. The United States FDA classified it as a weight loss drug, suitable to be consumed at a quantity of 10-15 mg per day. However in Thailand, sibutramine can be sold as a weight-loss drug to those who are obese or vastly overweight and are unable to lose weight by dieting or exercise, if they have no history of heart disease or respiratory problems. It can only be purchased with a prescription from a registered doctor [8-10].

Ephedrine (E) and **Pseudoephedrine** (PE) are generally the most abundant alkaloids found in Ephedra sinica. Ephedrine is a bronchodilator used to treat the symptoms of colds and asthma attacks. Pseudoephedrine is a milder stimulant than ephedrine and has been widely used as a nasal decongestant. Dietary supplements containing ephedrine, pseudoephedrine, either alone or in combination with other ingredients for losing weight, increasing energy and enhancing athletic performance could benefit for individually overweight people. The side effects include high blood pressure, irregular heartbeat, palpitations to stroke and even death [11-13].

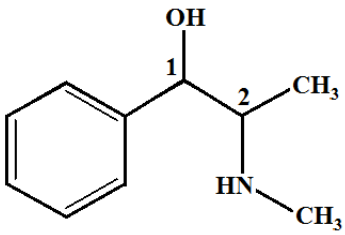
Fenfluramine (F) is related to enchanced serotonergic neurotransmission by facilitate the release of serotonin and inhibit reuptake by serotonergic nerve, resulting in a reduction in appetite. However, this drug has been withdrawn from markets and has not been used since 1997 in the US and European and since 2000 in Thailand due to its side effects of a risk of valvular heart disease, primary pulmonary hypertension, neurotoxicity and life threatening [14,15].



phentermine (pK_a 10.1)

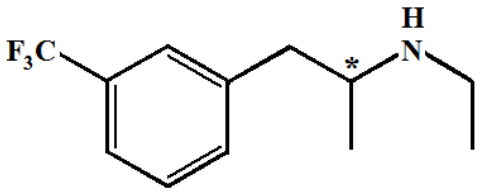


sibutramine (pK_a 8.5)



1R, *2S*- or *1S*, *2R*-ephedrine (pK_a 9.6)

1R, *2R*- or *1S*, *2S*-pseudoephedrine (pK_a 9.8)



fenfluramine (pK_a 9.1)

Figure 1.1 Chemical structures of five drugs.

1.2 Literature Review

Analytical separation approaches developed for determination of the anti-obesity drugs include gas chromatography (GC), high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE). Previous works on GC technique for different samples are for example, F in blood prepared by derivatization with heptafluorobutyryl-*S*-prolylchloride [16] or heptafluorobutyric anhydride [17] and in urine prepared by derivatization with *S*-(-)-*N*-(fluoroacetyl)-prolyl chloride [18], S in urine prepared by derivatization with *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide and *N*-methyl-bis-(trifluoroacetamide) asderivative [19], and E in supplement tablets prepared by derivatization with pentafluoropropionic anhydride [20].

In HPLC, the following works have been reported for determination of these drugs in a variety of samples with different sample preparations; E/PE in urine prepared by on-line column switching [21], F/P/E/other in blood prepared by derivatization with fluorescence substance and then extracted with solid phase extraction (SPE) [22, 23],

E/PE in herbs extracted by 9:1, MeOH:phosphoric and then cleaned up with SPE [24], S/F/other in supplement teas [25] and capsule [26] extracted by MeOH, PE/other in supplement tablets extracted by mobile phase [27], S/other in supplement powders extracted by MeOH [28] and in tablets extracted by MeOH [29], mobile phase [30] or 65:35 of MeOH:H_2O [31], E/PE in herbal medicine powders extracted three times with diethyl ether and then dissolved with mobile phase [32], S/F/other in supplement powders extracted twice with mobile phase using a ultrasonic bath [33], and E/other in capsule extracted with MeOH and followed by 9:1 n-pentane:diethyl ether and then dissolved with mobile phase [34].

Detection techniques used in HPLC include UV-Vis (HPLC-UV) [21, 24, 27-31] fluorescence (HPLC-FLD) [22, 23] or MS (LC-MS, LC-MS/MS) [25, 26, 32-34] HPLC-UV detection provided the limit of detection (LOD) as the followings; 5.5 and 13.2 μg/ml for E and S in urine, respectively [21], 2.29 and 0.09 μg/ml for S [30, 31] and less than 2 for S in supplement powders [28]. However, LOD can be improved from μg/ml (ppm) to ng/ml (ppb) levels by using HPLC-FLD; 0.8 and 0.45 ng/ml for S and F, respectively [23]. In addition, HPLC-MS or HPLC-MS/MS provided the comparable LOD with FLD; 1.8 and 27 ng/ml for F and S in supplement tea and capsule, respectively [25], and 1.0 and 14.2 ng/ml for F and S in supplement powders, respectively [33].

CE [35] is a separation technique in a capillary containing an electrolyte solution under the influence of an applied electric field. A CE separation mechanism is based on the difference in electrophoretic mobility of analytes. The simplest and most common type is capillary zone electrophoresis (CZE) that the capillary consists of a typical buffer such as borate, phosphate or acetate. Detector commonly used in CE is UV-Vis. Previous CZE works on determination of these drugs are for example; F/P/other substances in herbal powder and capsules for weight loss [36], E/other in illicit tablets [37], supplement capsules and herbal medicine powder [38], E/PE in water and urine [39].

As can be seen in Figure 1.1, these drugs are weak base containing an amine functional group. In the presence of an acidic pH buffer, each of them can carry a positive charge, and is easy to be separated using CZE. Acidic buffers previously reported for CZE separation of these drugs include a pH 2 phosphate buffer containing 15% v/v ACN for P/F and other [36], a pH 3.0 phosphate buffer for E/amphetamine compounds [37]. In an alternative approach, a pH 9.2 borate buffer was reported for determination of E/other in herbal medicine powders and supplement capsules [38]. At this buffer pH within pK_a ± 1 for analytes, the analytes are in equilibrium of charge and uncharged form. In addition, cyclodextrins (CD) were added into the buffer for separation of enantiomers such as a pH 2.5 phosphate buffer containing 17.5 mM β-CD for separation of E/PE enantiomers in water and urine [39] or a pH 4.3 phosphate buffer containing 20 mM β-CD or 100 mM acetyl-β-CD, for separation of S enantiomers [40].

A simple sample preparation using a solvent extraction with a ultrasonic bath has been reported for CZE separation of the anti-obesity drugs; 15 min-extraction with H_2O for determination of E and amphetamine compounds in tablets [37] or 10 min-extraction with 10:1 v/v H_2O:MeOH for determination of E/other in supplement capsule and herbal medicine powders [37]. SPE, C8-SCX stationary phase and CH_2Cl_2:isopropanol:NH_3 (78:20:2 v/v) eluent, was also used for clean-up and preconcentration of F/P/other in herbal powder and capsules after sonicating the samples with H_2O using a 30 min-ultrasonic bath, and followed by evaporating the solvent and dissolving the residue with 50:50 v/v water:MeOH [36]. Headspace solid phase microextraction (HS-SPME), using a co-polymer (butyl-methacrylate) silicone fiber, was reported for determination of E/PE in urine [39]. Using UV detection in CE for these drugs, LODs were found the followings; 1 μg/ml for F [36], 0.4 μg/ml for P [36], and 1.4 μg/ml for E [36]. LOD may be improved by using HS-SPME with online column sample stacking for UV detection of, all E and PE down to 3 and 5 ng/ml, respectively [39]. In comparison with UV detector, laser fluorescence provided better LOD for detection of 4.8 and 1.6 ng/ml for E and PE, respectively [38].

Capillary electrophoresis-mass spectrometry (CE-MS) [39] has been developed as a powerful tool for the analysis of charged species. MS provides a higher potential for an identification and confirmation of components in complex matrix, information regarding the structures of the separated compounds, and high sensitivity detection. Application of CZE-MS used for analysis of pharmaceutical compounds are for example, drug discovery [40, 41], drug impurity profiling [42, 43], illicit drugs of abuse [44-46].

1.3 Aim and Scope

The main aim of this work is to optimize and validate a CZE-UV technique for simultaneous separation and determination of five anti-obesity drugs, F, P, E, PE and S adulterated in dietary supplements for weight control. CZE-UV separation of these five analytes will be performed using a 100 mM tris-phospate buffer at pH 2.5. The effect of the type and concentration of organic solvent affecting the resolution of five analytes will be investigated, using 0-40% ACN or 0-70% MeOH added in the buffer. Method validation will be evaluated using the following parameters: limit of detection (LOD), limit of quantization (LOQ), standard calibration plot, accuracy and precision. After that, the developed method will be used for determination of anti-obesity drugs in real samples of dietary supplements for weight control.

Another objective is to extend this work from CZE-UV to CZE-MS. Since MS requires a volatile buffer, preliminary study of CZE-UV separation will be performed using an ammonium acetate buffer containing 0-50% ACN or 0-70% MeOH. Suitable separation of five analytes in CZE-UV using this volatile buffer can be used in CZE-MS if the CE-ESI/MS instrument is available to operate in our laboratory. CE-ESI/MS can be used to confirm the anti-obesity drugs detected in the abovementioned real samples.

CHAPTER II
THEORY

2.1 Capillary Electrophoresis (CE) [35]

Capillary Electrophoresis (CE) is a separation technique for charged analytes in a capillary containing an electrolyte solution under the influence of applied electric field. A separation mechanism is based on the difference in electrophoretic mobility (μ) of analytes. This technique is widely applied with either organic or inorganic ions, pharmaceuticals, peptides, proteins, and polymers. The advantages of this technique include the simple sample preparation, short analysis time, low consumption of sample, buffer and organic solvent, and high efficient separation. Figure 2.1 shows a schematic diagram of basic CE instrument.

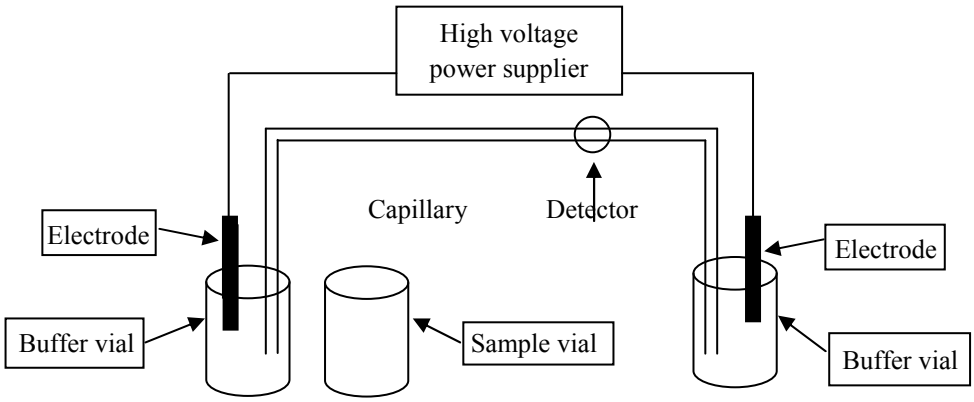


Figure 2.1 A schematic diagram of basic CE instrument. Adapted from Weinberger [47].

The basic CE instrumentation consists of a capillary, buffer and sample vials, a detector, a high voltage supplier and electrodes. A fused silica capillary is typically used with a 10 to 200 μm diameter (I.D.) and 20 to 100 cm in length. The outer capillary is coated by polymer, such as polyamide, for fragile protection. Two electrodes, such as platinum wire, are inert with chemical reactions. A typical buffer

used is for examples, phosphate, borate or acetate with or without organic solvent. The high voltage supplier is used in a range of -30 to 30 kV with maximum current up to 200 to 300 μA. The UV-Visible detector is commonly used for on-column detection, whereas, a mass spectrometric (MS) detector may be connected to an interface with the capillary end. The CE analysis is performed by filling the buffer in the capillary, and immersing capillary inlet and outlet ends together with electrodes. After the voltage is applied, the analytes migrate from the inlet end to the detection end and are separated due to their different velocities.

Six CE modes include; capillary electrophoresis (CZE), micellar electrokinetic chromatography (MECK), capillary electrochromatography (CEC), capillary gel electrophoresis (CGE), capillary isoelectric focusing (CIEF) and capillary isotachophoresis (CITP). This work involves only CZE, and therefore the CZE principle is described in the following section.

2.2 Capillary Zone Electrophoresis (CZE)

CZE is a basic technique and popular mode in CE due to its easy use and simple buffer, such as borate or phosphate as previously mentioned. From CZE separation mechanism as shown in Figure 2.2, analyte ions migrate under electric field to the electrode having opposite polarity with the analyte charge. At high electroosmotic flow (EOF) as described in Section 2.2.2, both anions and cations migrate to the detection window, while neutral molecules migrate due to EOF. Therefore, the CZE separation mechanism is only based on the differences in electrophoretic mobility of analytes, depending on the charge-to-size ratio of the analytes.

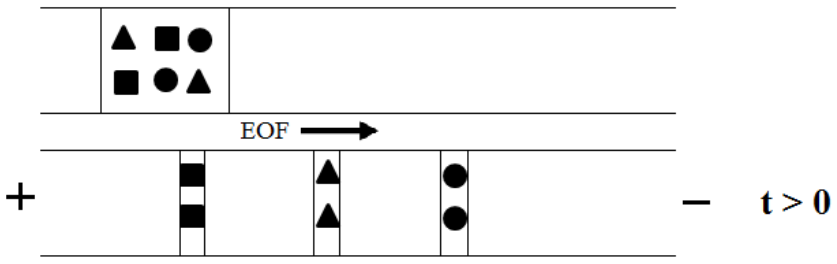


Figure 2.2 Separation mechanism in CZE.

2.2.1 Electrophoretic Mobility [48, 49]

Electrophoretic mobility of an analyte ion (μ, $m^2V^{-1}S^{-1}$) is defined as the electrophoretic velocity (v_{ep}) of the analyte ion per a unit of electric field strength (E), and related to parameters as given in Equation 2.1

$$\mu = \frac{v_{ep}}{E} = \frac{ze}{6\pi\eta r_h} \tag{2.1}$$

where z is the effective charge of the analyte ion, e is the charge of an electron (1.6×10^{-19}coulomb), η is the medium viscosity, and r_h is the hydrodynamic radius of the analyte ion surrounded with water and its counter ion when the analyte ion is migrating under the electric field.

The factors affecting a change in μ include buffer ionic strength, buffer pH, buffer viscosity, buffer temperature, and buffer additives. An increase in the ionic strength or the concentration of the buffer results in the larger number of counter ions surrounding the analyte ion. Due to the opposite charge between the counter ion and analyte ion, this reduces z and enlarges $r_{h,}$ and therefore decreases in the z/r_h ratio. It follows from Equation 2.1 that an increase in the ionic strength leads to a decrease in μ. A buffer pH affects the degree of dissociation (α) of the analyte that is weak acid or weak base. The effective electrophoretic mobility (μ_{eff}) of the analyte is directly proportional to α as given in Equation 2.2.

$$\mu_{eff} = \alpha\mu \tag{2.2}$$

From Equation 2.1, the μ value is inversely proportional to the buffer viscosity. The higher the buffer viscosity, the smaller the μ value. Since the viscosity is also inversely proportional to the temperature, the μ value is proportional to the buffer temperature. Organic solvent, such as MeOH and ACN, is one type of the buffer additives typically used in the CZE buffer. The μ value gradually decreases to the minimum with adding 0 to 20% v/v ACN or 0 to 50% v/v MeOH in the buffer, and then increases with the higher amount of these organic solvents. This is because the viscosity of the organic solvent/water increases to the maximum, and then decreases at the higher amount of organic solvents.

2.2.2 Electroosmosis [48, 49]

Electroosmotic flow (EOF) is another driving force in a CE capillary for movement of analytes. An inner surface of an uncoated fused silica capillary consists of silanol groups (Si-OH). At pH > 2, these silanol groups can be ionized to the negatively charged form on the capillary surface (-Si-O^-).

$$-Si - OH + OH^- \longrightarrow Si - O^- + H_2O$$

$$-Si - OH + H_2O \longrightarrow - Si - O^- + H_3O^+$$

The positively charged counter-ions from the buffer are attracted to the negatively charged wall, and the double layer occur. Some positively charged ions are formed at immobilized Stern layer at the inner surface wall, some at the diffusion layer adjacent to the Stern layer, and the rest counter-ions remain in the bulk solution. The Stern layer contains negative charges greater than positive charges. According to charge balance, the bulk solutions and the diffusion layer contains excess positive ions. When the voltage is applied across the capillary, ions together with water molecules migrate to the opposite polarity electrode. Due to the excess positive ions in the diffusion layer and the bulk solution, the water molecules or solution flow to the cathode. This phenomenon is called electroosmosis and this movement is called electroosmotic flow (EOF).

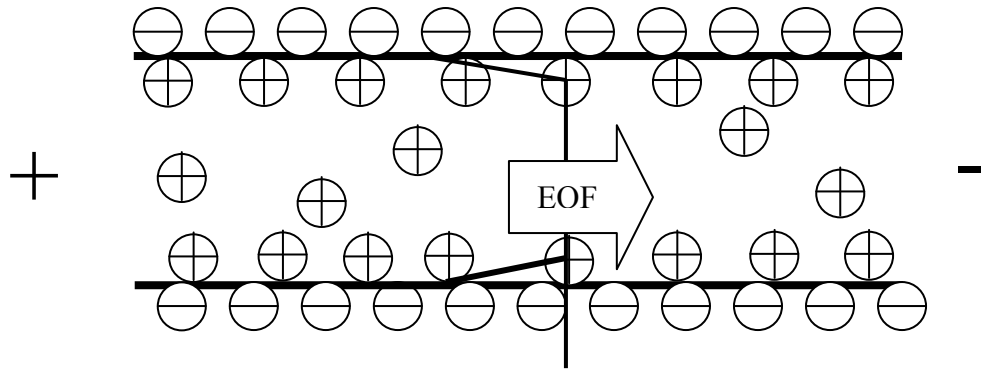


Figure 2.3 Electroosmotic flow (EOF).

Electroosmotic mobility or electroosmotic coefficient (μ_{eo}) is defined as the electroosmotic velocity (v_{eo}) per a unit of electric fieldstrength (E), and related to parameters as given in Equation 2.3

$$\mu_{eo} = \frac{v_{eo}}{E} = \frac{-\varepsilon_0 \varepsilon \zeta}{4\pi\eta} \quad (2.3)$$

where ε_0 is the permittivity of free space, ε is the dielectric constant and ζ is the Zeta electric potential [35].

The Zeta electric potential (ζ) is proportional to the thickness of diffusion layer (κ^{-1}) and charge density of the capillary charge (q^*, Cm^{-2}) as given in Equation 2.4.

$$\zeta \propto q^* \kappa^{-1} \quad (2.4)$$

The factors affecting a change in μ_{eo} include buffer pH, viscosity and temperature of buffer, concentration or ionic strength of the buffer, electric field or electric potential difference, and organic solvent. An increase in the buffer pH enhance a q^* due to ionization of –SiOH groups to $–SiO^-$ groups, leading to higher μ_{eo}. The viscosity and temperature of buffer affect a change in μ_{eo} in the same trend in μ as previously in Section 2.2.1. An increase in the concentration or ionic strength of a buffer reduces the diffusion layer thickness (κ^{-1}), leading to an increase in ζ, and then results in an increase in μ_{eo}. However, the too high concentration of the buffer may increase μ_{eo} due to Joule heating. The high electric field or electric potential difference applied may generate Joule heating, resulting higher μ_{eo}. Organic solvent added in the buffer decreases in μ_{eo} due to an increase in the viscosity near the capillary wall caused by the interaction between organic solvent molecules and capillary wall.

2.3 Migration Behaviour in CE [35]

The net mobility (μ_{net}) of the analyte ion is equal to the sum of its electrophoretic mobility (μ) and electroosmotic mobility (μ_{eo}) as given in equation 2.5.

$$\mu_{net} + = \mu + \mu_{eo} \quad (2.5)$$

At high EOF, all analytes migrate to the cathode end as shown in Figure 2.4. Each of the cations moves to the cathode with the same direction of its electrophoretic mobility and EOF. The higher the ion charge and the smaller the ion size, the faster the migration toward the cathode. Typically, anions have the direction of electrophoretic mobility to the anode, opposite direction with EOF. In the case of high

EOF, the positive value of the net mobility is obtained, indicating that the anions migrate toward the cathode. The higher the $|\mu|$ value, the longer the net migration. All neutral molecules move under EOF toward the cathode.

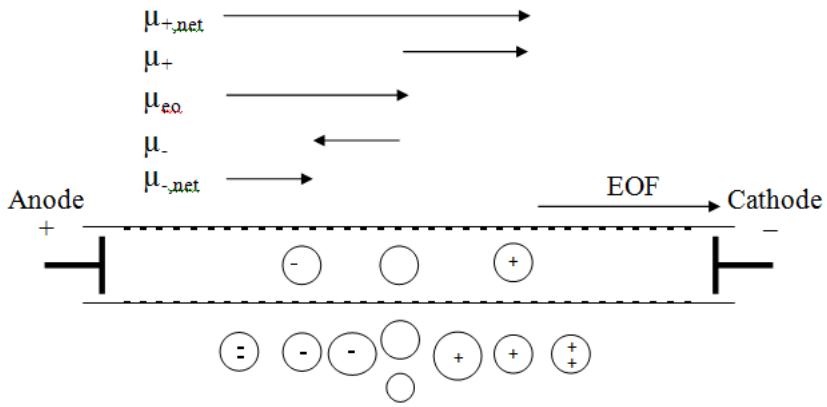


Figure 2.4 Migration behaviour of each species.

2.4 Electropherogram and Migration Time

When the analyte migrates toward the cathode, and the detector responses the analyte and generates signal as a function of time, called an electropherogram similar to chromatogram in chromatography. The duration of the analyte migrating from the inlet end of capillary to the detector is called migration time (t_m). From the electropherogram, the net mobility, electroosmotic mobility and electrophoretic mobility can be calculated using the following equations

$$\mu_{net} = \frac{lL}{Vt_m} \tag{2.6}$$

$$\mu_{eo} = \frac{lL}{Vt_{eo}} \tag{2.7}$$

$$\mu = \mu_{net} - \mu_{eo} = \left(\frac{1}{t_m} - \frac{1}{t_{eo}}\right)\frac{lL}{V} \tag{2.8}$$

where L is the total length of the capillary (m), l is the length from the inlet end of capillary to the detector (m), V is the electric voltage (Volt), t_{eo} is the migration time of neutral molecules or an EOF marker

2.5 Peak Dispersion in CE [35]

The idea peak in CE, similar to that in chromatography, has a Gaussian shape as shown in Figure 2.7. The peak consists of the standard deviation of the peak (σ), peak width at base (w) equal 4σ, the peak height (h) and the peak width at half height, ($w_{0.5h}$) equal 2.354σ

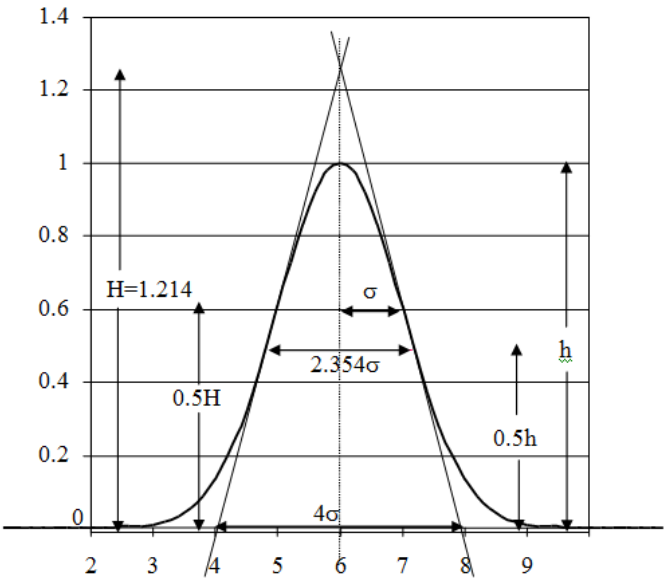


Figure 2.5 Guassian peak.

Contributions to the peak broadening are for examples: longitudinal diffusion, thermal dispersion, electromigration dispersion (EMD), and wall adsorption.

Longitudinal diffusion occurs the Guassian distribution as the same trend in HPLC distribute as given in Equation 2.9.

$$\sigma_{\text{diff}}^2 = 2Dt_{\text{m}} \tag{2.9}$$

where D is the longitudinal diffusion coefficient. σ_{diff}^2 is the peak variance caused by the longitudinal diffusion. The longer the migration time, the greater the peak broadening.

When the electric field is applied across a capillary containing a buffer, thermal energy is generated, called Joule heating. **Thermal dispersion** occurs by a parabolic

increase of buffer temperature from the inner wall to the middle capillary. This difference in temperature results in distribution of electrophoretic mobility of the analytes. The peak variance caused bythermal dispersion (σ_{th}^2) is given by the Equation 2.10.

$$\sigma_{\text{th}}^2 = 2D_{\text{th}}t_{\text{m}} \tag{2.10}$$

where D_{th} is the thermal diffusion coefficient. The longer the migration time, the greater the peak broadening.

$$D_{\text{th}} = \frac{f_{\text{T}}^2 \kappa_{\text{c}}^2 E^6 r_c^6 \mu^2}{3072\lambda_{\text{s}}^2 D} \tag{2.11}$$

where f_{T} is the factor of electrophoretic mobility elevation caused by a temperature increase, κ_{c} is the electric conductivity of the buffer, and E is the electric field applied. It can be seen from Equation 2.11 that great thermal dispersion may be due to high electric field, the large capillary diameter and the high electric conductivity or the concentration buffer.

Electromigration dispersion (EMD) occurs by different electrical conductivity of the analyte zone and the buffer zone. This results in triangular distribution of the peak, leading to tailing peak or fronting peak.

$$\sigma_{\text{EMD}}^2 = \left| \frac{2}{9} \mu E t_{\text{m}} l_{\text{inj}} C_{\text{A}} a_{\text{A}} \right| \tag{2.12}$$

where l_{inj} is the length of a sample solution injected, C_{A} is the analyte concentration, and a_{A} is the EMD factor that increases with a higher difference in the electrophoretic mobility of the analyte ion and buffer co-ion having the same charge, and with a lower concentration of the buffer. In order to minimize peak broadening due to EMD, the buffer co-ion used should have a similar μ value with the analyte ion, the length of the analyte solution injected should be minimize but the too short length gives low sensitivity detection, and the buffer concentration should be compromised because the high concentration minimizes EMD but generates greater Joule heating.

Wall adsorption produces tailing distribution especially due to the interaction between negatively charged capillary wall and positively charged analytes. The wall

adsorption can be minimized by using large capillary dimension and higher buffer concentration, but the higher Joule heating is obtained.

2.6 Resolution and Efficiency [35]

The resolution (R_s) for two analytes or peaks is defined as the difference in migration time (Δt_m) per the average ($\overline{w}$) of peak width as given in Equation 2.13.

$$R_S = \frac{\Delta t_m}{\overline{w}} \tag{2.13}$$

From Equations 2.5 to 2.8 and 2.13, and also the first paragraph of Section 2.5, the R_s value may be rearranged to relate to electrophoretic mobility, electroosmotic mobility and peak efficiency as given in Equation 2.14.

$$R_s = \frac{1}{4}\left(\frac{\Delta\mu}{\overline{\mu} + \mu_{eo}}\right)\sqrt{\overline{N}} \tag{2.14}$$

where $\overline{\mu}$ is average electrophoretic mobility of two analytes, $\Delta\mu$ is different electrophoretic mobility of the two analytes, and $\overline{N}$ is the average of peak efficiency or the number of theoretical plates which is defiled as the square of a ratio of the capillary length or migration time to the standard deviation of the peak as given in Equation 2.15

$$N = \left(\frac{l}{\sigma}\right)^2 = \left(\frac{t_m}{\tau}\right)^2 = 16\left(\frac{t_m}{w}\right)^2 \tag{2.15}$$

where l is the capillary length to the detector, σ and τ are the standard deviation of the peak in a unit of distance and time, respectively.

2.7 Qualitative and Quantitative Analysis in CE [50]

2.7.1 Peak Area and Corrected Peak Area

In qualitative analysis of CE with UV detection, each peak may be identified by comparing t_m and UV spectra of the analyte and standard, and using a spiking

technique to examine an increase in the analyte peak area before and after adding each standard into the sample solution for two consecutive runs.

For quantitative analysis, the amount of the analyte injected is related to the peak area of analyte, volume flow of the analyte migrating to a detector (V_F), and response factor as given in Equation 2.16.

$$Q(\text{mole}) = \frac{\text{Peak area} \times V_F(\text{m}^3\text{s}^{-1})}{\text{Response factor}\,(\text{AU mol}^{-1}\text{m}^3)} \tag{2.16}$$

The volume flow in a cylinder capillary with radius r_c is given by Equation 2.17.

$$V_F = \frac{\pi r_c^2 l}{t_m} \tag{2.17}$$

It follows from Equations 2.16 and 2.17 that Q is proportional to the ratio of the peak area and t_m.

$$Q \propto \frac{\text{Peak area (AUs)}}{t_m\ (\text{s})} \tag{2.18}$$

Unlike chromatography that the volume flow is equal for the analytes passing the detector at the flow cell, and therefore the analyte injected in chromatography is proportional to the peak area. In CE, t_m depends on both electrophoretic mobility and electroosmotic mobility. The analytes with equal amount and response factor may have different peak area due to the difference in electrophoretic mobility. For example, the faster the t_m value, the smaller the peak area. Therefore, CE quatitative analysis is generally performed by using the corrected peak area which is defined as the peak area divided by migration time (Equation 2.18).

2.7.2 Calibration Methods

Typically the amount of analytes in a sample may be determined using calibration methods such as external standard and internal standard. The external standard calibration is carried out by plotting a linear graph between corrected peak area (A_{corr}) of standard at various known concentrations against the standard concentrations, while the internal standard calibration is established by plotting A'_{corr} against the concentrations of each standard, where A'_{corr} is the ratio of A_{corr} for standard at various

concentrations and internal standard at a fixed concentration against the standard concentrations. Internal standard (ISTD) is another standard that has similar structure or similar functional group with the analytes, but neither exists in a sample nor interferes with detection of analytes. In multiple consecutive runs with the same concentration, the amount of standard injected may be varied, while the amount ratio for standard and internal standard should be the same. This implies the higher precision in A'_{corr} than A_{corr}. Therefore, the internal standard method should provide higher precision in quantitative analysis.

2.8 Capillary Electrophoresis-Mass Spectrometry (CE-MS) [39, 51]

A mass spectrometer (MS) can be coupled with CE and used as a detector instead of a UV detector. MS including tandem MS can improve detection sensitivity, confirm the analyte mass from mass-to-charge ratios (m/z) and mass spectrum, and provide structural information of the analytes and unknown compounds. The schematic diagram of a CE-MS systemis shown in Figure 2.8. When analytes in a buffer from a CE outlet end enter a CE-MS interface, they become gas phase ions in an ionization source or an ion source by evaporating solvent molecules. The gas phase ions are then detected in a mass analyzer by measuring the mass-to-charge ratios (m/z) to generate mass spectrum, a plot of intensity or relative amount of ions as a function of m/z.

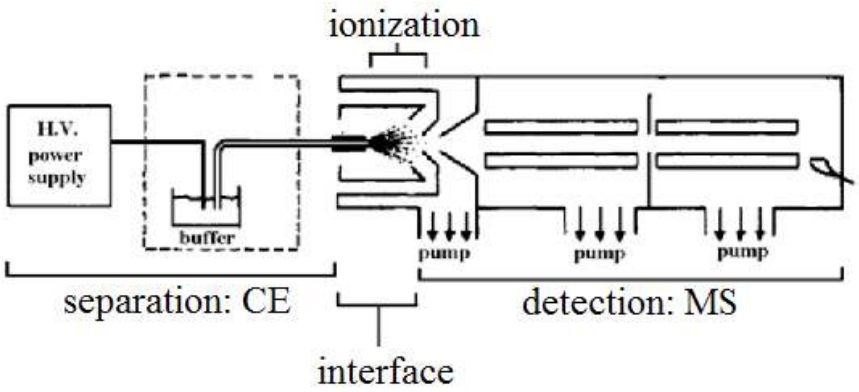


Figure 2.6 A schematic diagram of CE-MS system [52].

2.8.1 Interfaces [52]

Two types of CE-MS interfaces, developed from LC-MS interfaces, include sheath-flow and sheathless interfaces with addition of a make-up liquid and without additional liquid, respectively. The sheathless interface provides better sensitivity due to no dilution of the analytes from an additional liquid.

2.8.1.1 Sheath-Flow Interface

A make-up liquid added into the interface side is aimed to adjust the interface between the capillary end and the ion source to have proper electrical connection and flow rate of an analyte solution. This improves the convertion of analytes from a liquid phase into a gas phase, and decreases the formation of undesired droplets at the outlet end of the CE capillary because the droplets cause an unstable flow and unstable spary. There are two types of sheath-flow interfaces: the coaxial sheathliquid interface and a liquid-junction interface. In a coaxial configuration, liquid addition takes place proximal to MS, while the liquid-junction geometry provides the make-up liquid distal to the sprayer tip.

2.8.1.2 Sheathless Interface [53]

The sheathless interfaces can be performed by connecting a nanospay needle with the outlet end capillary, coating the outlet end capillary tip as an emitter with conductive, or inserting a conductive wire into the outlet end capillary. Sensitivity enhancement in sheathless interfaces is resulted from a nanospray tip positioned more closely to the MS than other interfaces, improvement of analyte ionization due to smaller droplets obtained, and no sample dilution by an additional liquid.

2.8.2 Ionization Sources: Electrospray Ionization (ESI) [54]

Three main ionization sources of mass spectrometry applied in CE consist of electrospray ionization (ESI), continuous flow-fast atom bombardment (FAB) and

matrix assisted laser desorption ionization (MALDI). However ESI is widely used, and therefore described in this work. ESI is a soft ionization source at an atmosphere pressure for on-line CE-MS, and a suitable method for the analysis of ionizable or polar compounds. An analyte solution is sprayed to form small droplets, and the solvent is evaporated. Each of the small diameter droplets with unchanged charges results in an increase in charge density and finally becomes smaller droplets with a single charge before moving to the mass analyzer. Several advantages of ESI include simplicity, high ionization efficiency, and ability to produce multi-charged ions. However, the main limitation of CE-ESI/MS is that the volatile buffers required such as formate, acetate, carbonate, and ammonium. In addition, low concentration of the buffer should be used in order to avoid interferences with ionization of the analyte.

2.8.3 Mass Analyzer [51]

Mass analyzers used in HPLC can be coupled with CE and are divided into two categories, ion transmission and ion trapping systems. For the ion transmission system such as a quadrupole, time of flight and a magnetic sector, separation ions from anion source move through a mass analyzer and a detector. For ion trapping system, ions from an ion source are trapped in amass analyzer before passing to a detector, such as ion trap and fourier-transform ion-cyclotron resonance.

2.8.3.1 Quadrupole Mass Analyzer

The quadrupole mass analyzer is used mainly for CE-MS due to relatively low cost, compact size, and easy to operate. The quadrupole analyzer consists of four metal rods which must be parallel. The each opposite rods pair is connected with a radio frequency (RF) and a direct current (DC), respectively. When ions enter between the rods of the quadrupole, ions are vibrated by particular RF and DC applied, in order to select ions of a certain mass-to-charge ratio (m/z). Only selected ions or resonance ions pass through the quadrupole analyzer, while unstable ions are destroyed by colliding with the rods. By continuously varying the applied voltage, the selected ions with a wide range of m/z are obtained.

2.8.3.2 Time of Fight (TOF) Mass Analyzer

A TOF analyzer is the most notable technique developed for detector in CE. Moving ions are accelerated toward the detector by a fixed potential. After acceleration, ions with same charges travel with same kinetic energy, but different velocity depending on m/z. The heavier the ion, the longer the time of fight. TOF analyzer can generate mass spectra quickly with high sensitivity. Advantages of TOF include high mass resolution, high mass accuracy and a wide range of mass.

2.8.3.3 A Quadrupole Time-of-Fight (Q-TOF) Tandem Mass Spectrometer [55]

A quadrupole time-of-fight (Q-TOF) tandem mass spectrometer (MS/MS) consists of a quadrupole mass analyzer, a quadrupole collision cell and a time-of-fight mass analyzer. Ions generated from an ion source are transferred to the quadrupole analyzer (MS1) and then precursor ions with a certain m/z are obtained. A collision cell between these two mass analyzers is used to produce fragment ions. After that, fragment ions are focused onto the pusher by the acceleration towards the reflectron to reflect ions to a detector to obtain a mass spectrum.

CHAPTER III
EXPERIMENTAL

3.1 Chemicals and Materials

Standards: sibutramine (S), phentermine (P), fenfluramine (F), ephedrine (E), pseudoephedrine (PE) were purchased from Sigma-Aldrich (Steinheim, Germany). Ethanol (EtOH), methanol (MeOH), phosphoric acid and hydrochloric acid were obtained from Merck (Darmstadt, Germany); acetonitrile (ACN) and tris-(hydroxymethyl) aminomethane from Sigma-Aldrich (Steinheim, Germany); sodium hydroxide from Labscan Asia (Thailand) Co, LTD. Internal standard (IS) is *p*-toluidrine, was obtained from Sigma-Aldrich (Steinheim, Germany). The real samples were purchased from an online market (Thailand) and their brand names cannot be disclosed.

3.2 CE Conditions

All CE experiments were performed on an MDQ Beckman CE Instrument (Beckman Coulter, Inc., CA). An uncoated fused silica capillary used was 50 μm i.d. × 60.2 cm (50 cm to detector). The temperature was maintained at 25 ^{o}C. The following conditions were used for CE analysis; voltage of +25 kV, 0.5 psi pressure injection for 10 s, and photo diode array-UV detection by scanning in a range of 200-400 nm and monitoring at 214 nm. Prior to analysis for each day, the capillary was rinsed with 0.1 M phosphoric acid for 10 min followed by the running buffer for 10 min. Between each injection, the capillary was rinsed with 0.1 M phosphoric acid and a running buffer for 3 min. After analysis for each day, the capillary was rinsed with 0.1 M NaOH for 10 min and then water for 10 min.

3.3 Preparation of Buffer for CE-UV

A pH 2.5 tris-phosphate buffer is prepared by titrating a 500 mM phosphoric acid solution with a 5.0 M tris-(hydroxymethyl) aminomethane solution. Then the buffer was diluted five-fold with Milli-Q water and with or without ACN or MeOH to give a 100 mM phosphate buffer. All running buffers were filtered through 0.45 μm filters, and then sonicated in an ultrasonic bath for 10 min prior to use for CE analysis. A 100 mM phosphate buffer at pH 2.5 contains an appropriate type and amount of organic solvent. The organic solvent, such as ACN and MeOH at 0 to 40% v/v and 0 to 70% v/v, respectively, was separately added in a pH 2.5 tris-phosphate buffer. Results and discussion are in Section 4.1.4.

3.4 Preparation of Standard Solutions

Stock standard solutions at 1000 ppm each in 4:6 MeOH:Milli-Q water were separately prepared by dissolving solid standards with MeOH and then diluting these with Milli-Q water. Working standard solutions containing five standards were prepared by pipetting an appropriate amount of each stock standard solution and then diluting these with Milli-Q water.

3.5 Validation of CZE-UV Method [56]

In this section, a pH 2.5 100 mM tris-phosphate buffer containing 20% v/v ACN was used, by using *p*-toluidine as an internal standard and other conditions as previously mentioned in Section 3.2.

3.5.1 Limit of Detection (LOD) and Limit of Quantization (LOQ)

Various levels of diluted concentration of the five standards from each stock solutions were prepared and the ratios of signal-to-noise (*S/N*) were determined. The limit of detection and quantitation were obtained from the five standard concentrations giving the *S/N* of 3 and 10, respectively. Therefore, the calculated LOD and LOQ are

obtained from $3C_D/S_D$ and $10C_Q/S_Q$, repectively, where C_D and C_Q are concentrations of standrad near LOD and LOQ providing the average peak height at approximately 3 and 10 times over the baseline noise (S_D and S_Q), repectively. Results are given in Section 4.2.1.

3.5.2 Calibration Plot

The working standard solutions containing five standards in a concentration of LOQ to 50 ppm were prepared by pipetting the appropriate amounts of each stock standard solutions each as presented in Table 3.1, and then diluting the mixed these solution with Milli-Q water to have the final volume of 1500 µL. It should be noted that each final standard solution contained 10 ppm *p*-toluidine internal standard and 10% v/v of buffer. At each concentration, peak areas of all five standards were obtained from the duplicate runs. A calibration plot for each analyte was obtained by constructing a linear plot of A'_{corr} against its concentrations, where A'_{corr} is the ratio of corrected peak area of the standard to that of the internal standard. Results are discussed in Section 4.2.2.

Table 3.1 Concentration levels of mixed standards (ppm) for calibration plot and linearity

Analytes	Level 1	Level 2	Level 3	Level 4	Level 5	Level 6	Level 7
P	0.45	1.0	2.0	5.0	10.0	25.0	50.0
PE	0.50	1.0	2.0	5.0	10.0	25.0	50.0
E	0.50	1.0	2.0	5.0	10.0	25.0	50.0
F	0.55	1.0	2.0	5.0	10.0	25.0	50.0
S	1.00	1.0	2.0	5.0	10.0	25.0	50.0

3.5.3 Accuracy and Precision

Accuracy and precision of standards in the matrix (water) for quantitative analysis was assessed at three levels of LOQ, 5 and 30 ppm as presented in Table 3.2. Intraday precision, the average and relative standard deviation (RSD) of t_m and the measured concentration of five analytes were evaluated from five consecutive runs, while

interday precision was evaluated from five consecutive days and each day for five runs. The measured concentration was obtained from A'_{corr} and a calibration plot of each analyte. Results are discussed in Section 4.2.3.

Table 3.2 Concentration levels of mixed standards (ppm) for the study of accuracy and precision

Analytes	Level 1	Level 2	Level 3
P	0.45	5.0	30.0
PE	0.50	5.0	30.0
E	0.50	5.0	30.0
F	0.55	5.0	30.0
S	1.00	5.0	30.0

3.6 Application to Real Samples [37]

Each representative powder sample was obtained from 12 capsules and its average weight per capsule was determined. An appropriate amount of homogeneous powder sample was weighted, and then extracted with a 1.0 ml of 10:1 Milli-Q water:EtOH by sonicating this in an ultrasonic bath for 10 min, and then 12,000 ppm centifuging for 10 min at room temperature. Prior to CE analysis, each aliqout was filtered through a 0.45 μm filter, and appropriately diluted with 10% buffer.

3.7 CZE-UV Separation Using a Volatile Buffer for MS Detection

An ammonium acetate buffer is prepared from a stock solution of a 100 mM ammonium acetate solution at a pHs 4, 5 and 6, respectively. Then the buffer was diluted five-fold with Milli-Q water and with or without ACN or MeOH to give a 20 mM ammonium acetate buffer. All running buffers were filtered through 0.45 μm filters, and then sonicated in an ultrasonic bath for 10 min prior to use for CE analysis. An ammonium acetate buffer at pHs 4, 5 and 6 contains appropriate type and amounts of organic solvent. The organic solvent, such as ACN and MeOH at 0 to 50% v/v and 0 to 70% v/v, respectively, were separately added in an ammonium acetate buffer at

desired pH. Other CE conditions are in Section 3.2. Results and discussion are in Section 4.4.

3.8 ESI-MS and ESI-MS/MS Conditions

All ESI-MS experiments were performed on a micrOTOF-Q Bruker Instrument (Bruker Daltonics Inc., Germany). In this work, structural information of the analytes was determined by ESI-MS using a quandrupole mass analyzer and ESI-MS/MS using quadrupole time-of-flight mass analyzers, along with a positive ESI mode and direct injection with a syringe pump. The first quandrupole was scanned to obtain precursor ions with a certain mass in a range of 50 to 1000 m/z. In a range of 5-15 eV, the collision energy was found to generate highest intensity of product ions. Other the acquisition parameters for ESI-MS and ESI-MS/MS are shown in Table 3.3.

Table 3.3 Acquisition parameters for ESI-MS and ESI-MS/MS

Source Type	ESI
Scan Begin	50 m/z
Scan End	1000 m/z
Ion Polarity	Positive
Set Capillary	4500 V
Set End Plate Offset	-500 V
Set Collision Cell RF	150.0 Vpp
Set Nebulizer	0.3 Bar
Set Dry Heater	180 °C
Set Dry Gas	4.0 l/min
Set Divert Valve	Waste

CHAPTER IV
RESULTS AND DISCUSSION

4.1 CZE-UV Conditions and Optimizations

4.1.1 pH, Concentration and Type of Buffer

The five anti-obesity drugs are weak base containing an amine group with their pK_a in a range of 8-10 as shown in Figure 1.1. In an aqueous solution, the dissociation and protonation of weak base, B, are expressed as Equations 4.1 and 4.2, respectively.

$$B + H_2O \overset{K_b}{\rightleftharpoons} BH^+ + OH^- \text{ or } BH^+ + H_2O \overset{K_a}{\rightleftharpoons} B + H_3O^+ \quad (4.1)$$

$$B + H_3O^+ \rightleftharpoons BH^+ + H_2O \quad (4.2)$$

The degree of ionization (α) of the weak base is related to the concentrations at equilibrium in Equation 4.3, which may be rearranged to relate to pH and pK_a as given in Equation 4.4 [48].

$$\alpha = \frac{[BH^+]}{[B]+[H_3O^+]} \quad (4.3)$$

$$\alpha = \frac{1}{1+10^{(pH-pK_a)}} \quad (4.4)$$

It follows from Equations 4.2 and 4.4, that in an acidic buffer especially with pH < pK_a at least 2 units, almost analytes carry a net fully positive charge ($\alpha \geq 0.99$). In this work, therefore, an acidic phosphate buffer at pH 2.5 was chosen in order to perform CZE separation of positively charged analytes, due to acceptable buffering capacity and the low UV-absorbing phosphate buffer in a wavenumber range of 200-300 nm. It should be noted that, a pH 2.5, the phosphate buffer contains weak acid H_3PO_4 (HA) and conjugate base $H_2PO_4^-$, (A^-), and the buffer pH is expressed by the Hednerson-Hasselbalch equation [57]

$$pH = pK_a + \log\frac{[A^-]}{[HA]} \quad (4.5)$$

where [HA] and [A^-] are the concentrations of weak acid (H_3PO_4) and conjugate base ($H_2PO_4^-$), respectively, and K_a is the acidic dissociation constant (K_{a1} for H_3PO_4). In order to maintain buffering capacity, the buffer pH should lie within the range of pK_a± 1 [35], where pK_{a1} is 2.12 for H_3PO_4 [58]. Strictly speaking, the buffer pH at pK_a provide the high buffering capacity, but low pH, especially near 2.0, is not suitable for the glass capillary wall. However, the high pH increases the adsorption of positively charged analytes on the negatively charged capillary wall. Therefore, the buffer at pH 2.5 was chosen to maintain the buffering capacity and minimise the analyte-wall adsorption.

In this work, a tris-H^+ co-ion was used in order to reduce peak distortion caused by electromigration dispersion (EMD) as previously mentioned in Section 2.5. This is becausethe tris-H^+ co-ion has electrophoretic mobility matching with five analytes ions (μ= 2.9× $10^{-8}$for the tris-H^+ ion [59], while μ = 1.80-2.30 × $10^{-8}m^2V^{-1}s^{-1}$ the five analytes ions measured in this work as shown in Figure 4.2)

Typically, the low concentration of the buffer will result in peak distortion caused by EMD and analyte-wall adsorption, while the very high concentration leads to Joule heating. In order to compromise between small EMD/wall adsorption and low Joule heating, the 100 mM phosphate buffer was used in this work, according to previously works on CZE separation of positively charged analytes using the low acidic phosphate buffer [47].

4.1.2 The Separation of Five Analytes Using 50 μm I.D. Capillary

An uncoated fused silica capillary with 50 μm internal diameter (I.D.) × 60.2 cm in length (50 cm to detector) is used in the analysis of anti-obesity drugs. Normally, a capillary of either 50 or 75 μm is used. A 75 μm capillary gives higher sensitivity and lower interaction between the five analytes with wall capillary, but it may cause lower resolution due to peak broadening caused by Joule heating. In addition, A 75 μm I.D. capillary has limitation of the use of high voltage and high concentration of buffer. A 75 μm I.D. capillary should be used to improve sensitivity and to decrease interaction

with the wall capillary, when the separation has no effect on the resolution. Otherwise, a capillary of 50 μm has an advantage because it decreases Joule heating, which can lead to reduction in peak broadening [47]. In addition, it was also used for many applications due to its compromise among resolution, sensitivity, heat dissipation and interaction with the capillary wall. Therefore, 50 μm I.D. capillary was selected for determination of five anti-obesity drugs.

4.1.3 Wavelength of Detection

The UV spectra of the five analytes inthe range of 200-300 nm with the maximum absorbance at 214 nm as shown in Figure B-1 in Appendix B. Electropherograms were monitored at wavelengths of 214 and 254 nm. This wavelength will be applied for the quantitative analysis of five analytes due to its low noise and high sensitivity.

4.1.4 The Effect of the Organic Solvent

In preliminary study, CZE separation of five analytes of our interest was performed using a 100 mM phosphate buffer at pH 2.5, but first two analytes were co-migrated. Typically, adding organic solvent in the buffer affects a change in electrophoretic mobilities of analytes [48], and may lead to improve simultaneous separation of several analytes. Therefore, effects of the type and concentration of organic solvent on a change in the migrating behavior and separation of the five analytes were investigated using 0 to 50% ACN and 0 to 70% MeOH as mentioned in Section 3.3, and CZE electropherograms are shown in Figure 4.1.

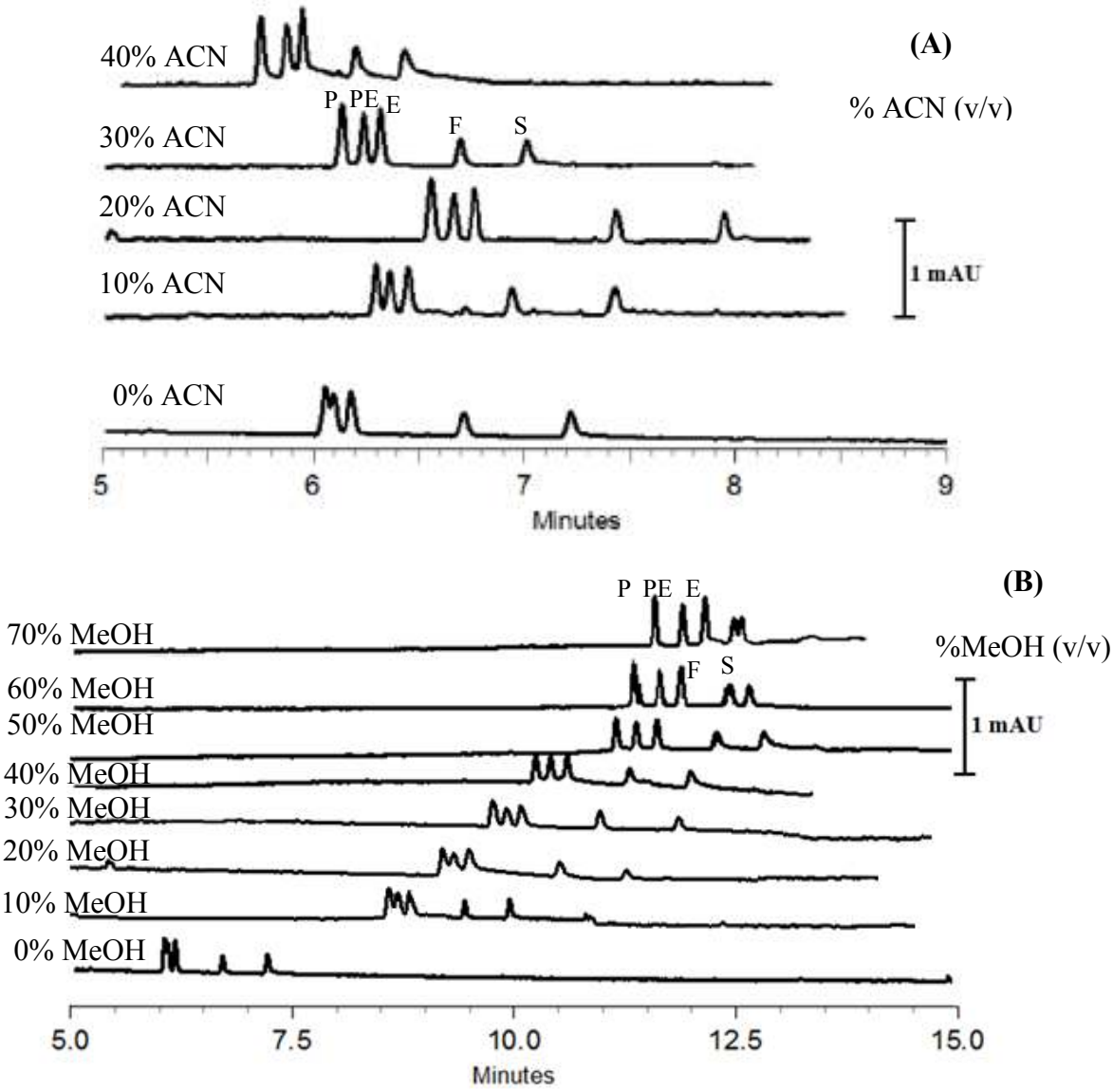


Figure 4.1 CZE electropherograms of five analytes using organic solvents, (A) 0-40% ACN, (B) 0-70% MeOH, added into a 100 mM tris-phosphate buffer at pH 2.5. CE conditions: uncoated fused silica capillary 50µm i.d.× 60.2 cm (50 cm to detector), temperature 25 ^{o}C, voltage of +25 kV, 0.5 psi pressure injection for 10 s and UV detection at 214 nm.

Identification of peaks in the electropherograms was performed by a spiking technique and UV spectrum comparison of each peak and each standard. The migration order, from the first to the last, was found to be P, PE, E, F and S, respectively. In the buffer at pH 2.5 with a suppressed EOF condition, the migration order mainly depends on electrophoretic mobility (µ) of analyte. The higher the electrophoretic mobility, the faster the migration order. As can be seen in Figure 4.1, for example, P has fastest migration, which is consistent with Equation 2.1 ($\mu \propto 1/\gamma_h$)

and their chemical structures shown in Figure 1.1 that P has smallest structure. Meanwhile, the last migration order of S was obtained due to its largest structure among these five analytes.

The effect of organic solvents on a change in migration behavior is first discussed, such as electroosmotic mobility (μ_{eo}), electrophoretic mobility (μ) and net mobility (μ_{net}), where $\mu_{net} = \mu_{eo} + \mu$, and all mobilities are calculated using Equations 2.6-2.8. Form Figure 4.2, a decrease in μ_{eo} was obtained with an increase in 0 to 70% MeOH while μ_{eo} was found to be slightly different for 0 to 20% ACN and then to increase with an increase in ACN concentrations from 20 to 40%.It should be noted that $\mu_{eo} \propto \varepsilon\zeta/\eta$, as in Equations 2.3. An increase in amount of MeOH or ACN increases the viscosity η of a solution near the capillary wall but decreases the dielectric constant ε. By assuming that ζ slightly changes due to very small q^* caused by low dissociation of –SiOH to $–SiO^-$, a change in μ_{eo} in Figure 4.2 is found to be consistent with a change in the ε/η ratio that decreases from 90 to 61 for 100% water to 100% MeOH, but slightly increases from 90 to 110 for 100% water to 100% ACN [35].

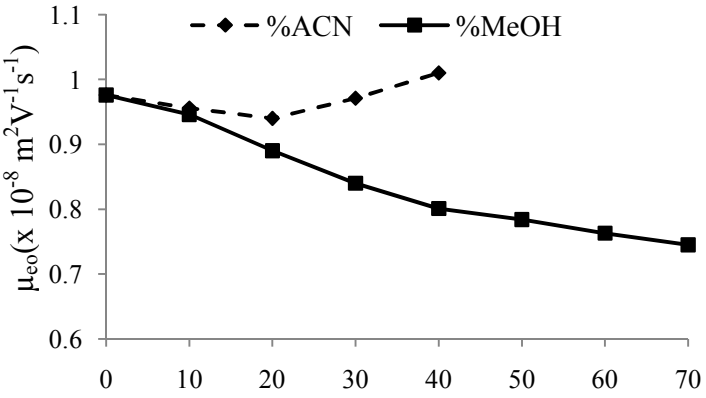


Figure 4.2 μ_{eo} in a 100 mM tris-phosphate buffer at pH 2.5 containing 0-40% ACN and 0-70% MeOH (v/v).

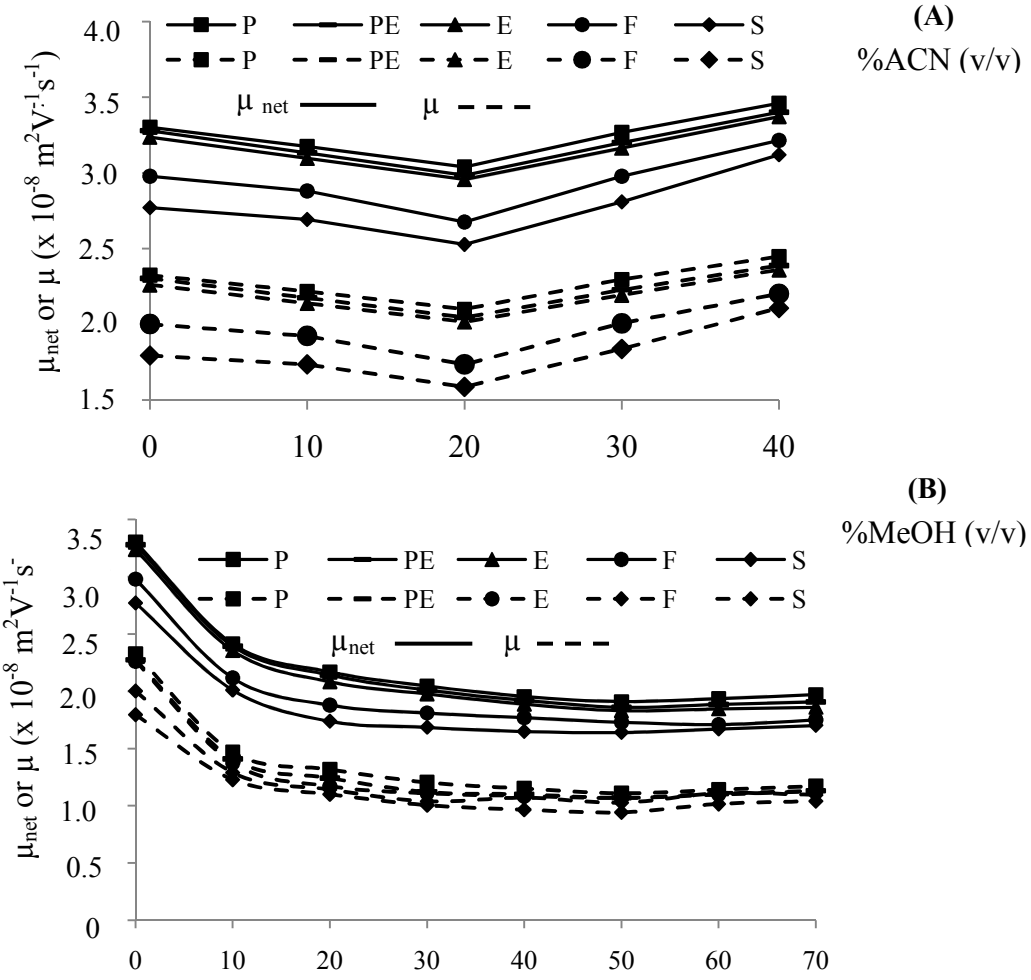


Figure 4.3 μ_{net} and μ in a 100 mM tris-phosphate buffer at pH 2.5 containing (A) 0-40% ACN, (B) 0-70% MeOH.

In the same trend with μ_{eo}, the μ value from Figure 4.3 decreases to the minimum with an increase in the concentrations of ACN at 20% or MeOH at 50%, respectively, and then slightly increases at higher concentrations of ACN or MeOH. This is consistent with an increase in the viscosity of the organic solvent/water to the maximum of ACN at 20% or MeOH at 50% as previously mentioned in the last paragraph of Section 2.2.1 ($\mu \propto 1/\eta$ as in Equation 2.1). Since the net mobility is the sum of the μ and μ_{eo} values that have the same direction of the vector and the same trend with an increase in the concentrations of ACN or MeOH. The μ_{net} value from Figure 4.3 decreases to the minimum, resulting in the longer t_m in Figure 4.1 ($\mu_{net} \propto 1/t_m$ as in Equation 2.6), to the with an increase in the concentrations of ACN at 20%

or MeOH at 50%, respectively, and then slightly increases at higher concentrations of ACN or MeOH.

In this work, the following factors are used to indicate the performance of the separation peaks: the resolution value (R_s), the efficiency or the number of theoretical plate (N), and the asymmetry factor (Asy). By considering the three closed peaks from selected electropherogrames in Figure 4.1, the calculated values of R_s and N as shown in Figure 4.4 are obtained using Equations 2.13 and 2.15, respectively. The asymmetry factor is defined as the ratio of a haft width at the 10% peak height from the baseline. The Asy factor values of 1.0, > 1.0 and < 1.0 indicate an ideal symmetry, tailing peak and fronting peak, respectively. In comparison between 20% and 30% ACN in Figures 4.4 (A) and (B), 20% ACN provided better R_s and N, and comparable Asy factor near 1.0. In comparison between 20% ACN and 60% MeOH in Figures 4.4 (A) and (C), 60% MeOH gave higher R_s but smaller N. This may be explained that the higher R_s with 60% MeOH is due to the greater $\Delta\mu$ or Δt_m ($R_s \propto \Delta\mu/\sqrt{N}$ as in Equation 2.14), while the lower N with 60% MeOH is due to the longer analysis time resulting in peaks broadening caused by longitudinal diffusion as in Equation 2.9. However, 20% ACN provides the higher peak height than 60% MeOH approximately 5-6 fold, implying better sensitivity using 20% ACN than 60% MeOH. Therefore, in order to achieve fast analysis time, baseline resolution for the quantitative analysis (R_s> 2.0), good asymmetry (nearly 1.00) and high sensitivity, 20% ACN, together with a 100 mM phosphate buffer at pH 2.5, was chosen for a suitable condition of simultaneous separation of five analytes, and for validation of the method and application to real samples in the following sections.

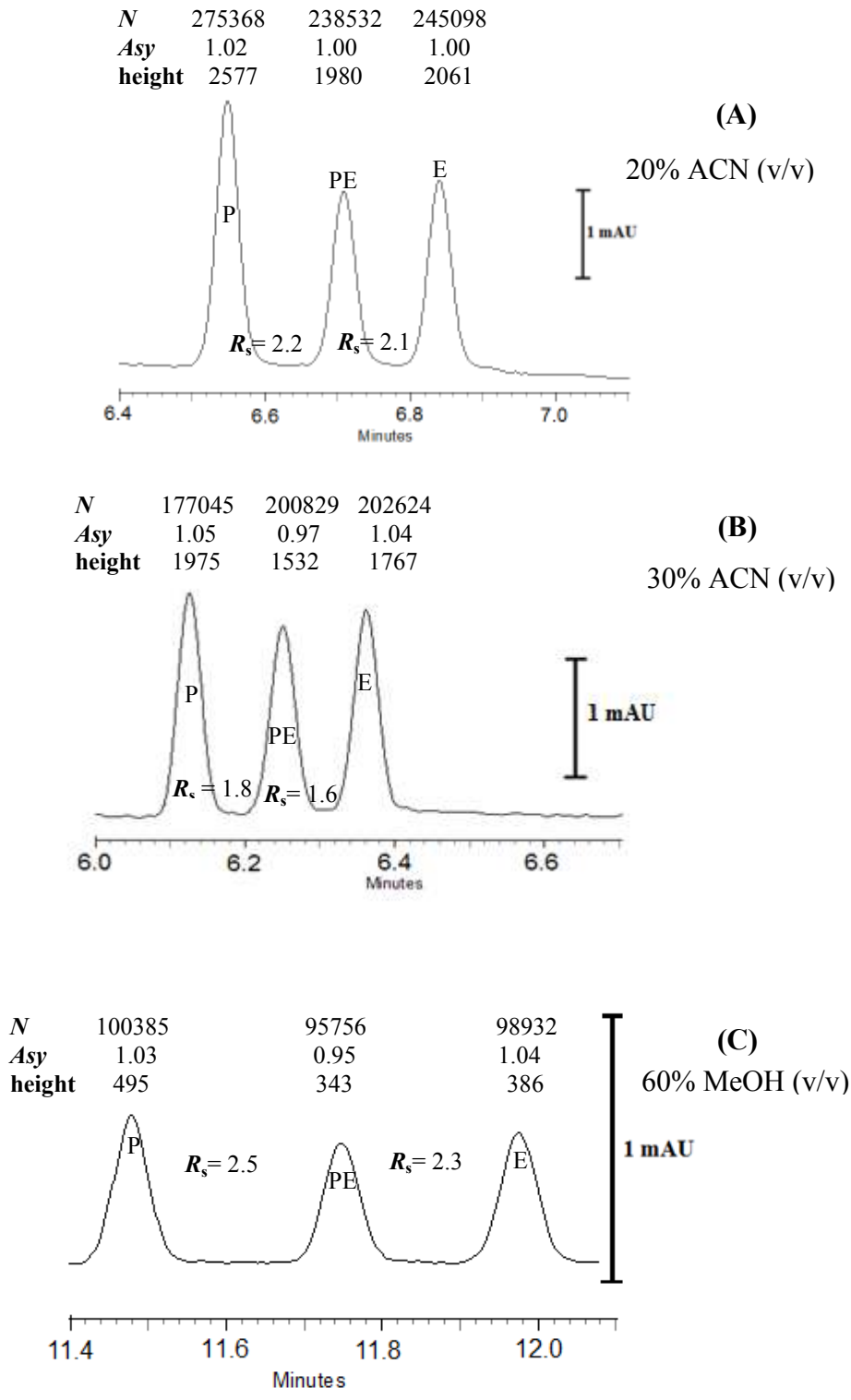


Figure 4.4 Electropherograms of P, PE and E, respectively, showing *N*, *Asy* and R_s values using a 100 mM tris-phosphate buffer at pH 2.5 containing (A) 20% ACN, (B) 30% ACN and (C) 60% MeOH.

4.2 Validation of the CZE-UV Method

4.2.1 Limit of Detection (LOD) and Limit of Quantitation (LOQ)

LOD and LOQ [60] are typically obtained from the concentrations of the analyte providing signal-to-noise ratios (*S/N*) of 3 and 10, respectively (Section 3.5.1). As shown in Table 4.1, instrumental LOD and LOQ for five analytes were obtained in a range of 0.10 to 0.20 ppm and 0.45 to 1.00 ppm, respectively. In this work, the samples are in a solid form, therefore, method detection limit (MDL) and method quantitation limit (MQL) are described by the equation MDL = LOD × *V/w* and MQL = LOQ × *V/w* (Table 4.1), where *w* is the sample weight for determination, and *V* is the final volume of sample in form solution (1.0 mL). If the sample is weighed with the different amounts, MDL and MQL will depend on the sample weight *w*. For *w* of 0.25 g, the values of MDL and MQL shown in Table 4.1 sufficiently allows to measures the five analytes intently adulterated in the sample.

Table 4.1 Limit of determination of five analytes.

Analytes	Instrumental (standards solution)		Real samples (weighting w g with solvent 1.0 mL)			
	LOD (ppm)	LOQ (ppm)	MDL (ppm)		MQL (ppm)	
			w	w = 0.25 g	w	w = 0.25 g
P	0.15	0.45	0.15/w	0.60	0.45/w	1.8
PE	0.15	0.50	0.15/w	0.60	0.50/w	2.0
E	0.15	0.50	0.15/w	0.60	0.50/w	2.0
F	0.10	0.55	0.10/w	0.40	0.55/w	2.2
S	0.20	1.00	0.20/w	0.80	1.00/w	4.0

4.2.2 Calibration Plot

Internal standard calibration, as previously detailed in Section 2.6.2, was chosen in this work. *p*-Toluidine was used as an internal standard because it contains an amine group similar to five analyts, absorbs a UV spectrum in a same range of the five analytes and does not interfere with detection of five analytes. The calibration was

obtained by constructing linear plots of the A'_{corr} against the concentrations of each standard at seven levels ranging from LOQ to 50 ppm, where A'_{corr} is the ratio of corrected peak area of the standard to that of the *p*-toluidine internal standard (see experimental in Section 3.5.2). From the calibration plots in Figure 4.5, the values of slope, intercept, and coefficient of determination (r^2) are summarized in Table 4.2.The linear relationship was obtained with r^2 ranging from 0.9990 to 0.9998.

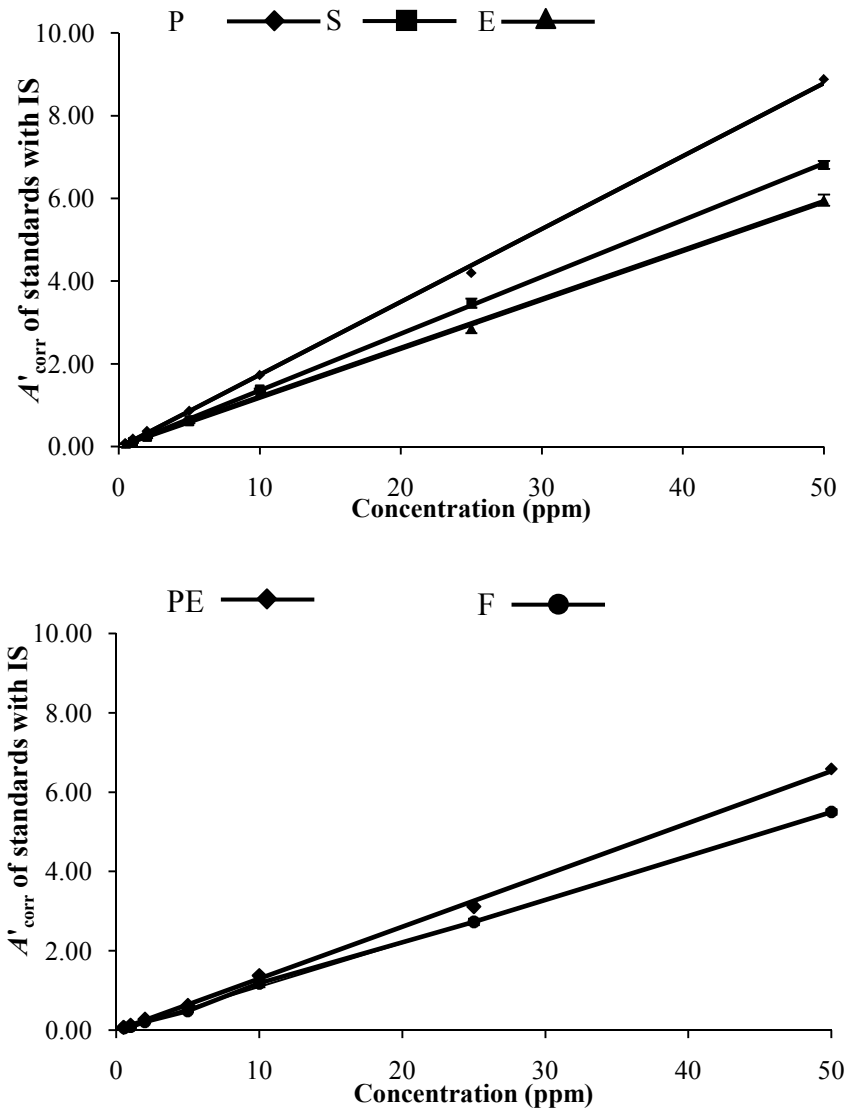


Figure 4.5 Calibration plots of five standards.

Table 4.2 Slope, intercept, and coefficient of determination from standard calibration plot of five standards.

Analytes	Concentration range (ppm)	Calibration plots		
		Slope	Intercept	r^2
P	0.45-50.00	0.1762	-0.0227	0.9993
PE	0.50-50.00	0.1307	-0.0105	0.9991
E	0.50-50.00	0.1182	0.0131	0.9990
F	0.55-50.00	0.1103	-0.0120	0.9996
S	1.00-50.00	0.1373	-0.0200	0.9998

4.2.3 Accuracy and Precision

Precision [60] is the closeness of agreement between measured quantity values obtained by replicate measurements on the standards under specified conditions. In this work, the intraday and interday precisions in t_m and the measured concentration were investigated at the three levels, LOQ, 5 and 30 ppm, of mixed standards spiked in a 10% diluted buffer. The intraday precision was obtained from five consecutive runs, while the interday precision from five consecutive days and each day for five runs. The RSD values of t_m and the measured concentration for intraday and interday precisions were evaluated using statistical ANOVA: Single Factor analysis at the 95% confidence level. An overall %RSD value is calculated using %RSD = $100S_r/\bar{x}$, where $\bar{x}$ is the average and S_r is the square root of the within group mean square value (*MS*) obtained from the ANOVA data as shown in Table A-1 and A-2 in Appendix A. If the *p*-value is greater than 0.05, non-significant difference in RSD for five consecutive days is obtained. This refers that %RSD for interday precision is equal to overall %RSD. In the other hand, if the *p*-value is less than 0.05, significant difference in RSD is obtained. In this work, the *p*-values more than 0.05 were obtained for all data sets. From the Table 4.3, high precision in t_m was obtained with RSD < 1%. However, a slight increase in t_m for interday is possibly owing to the variation in chemistry properties of capillary wall surface each day, resulting in changes of EOF and retention time of the standards. In Table 4.4 for all three concentration levels, acceptable precision in the measured concentration was also obtained with the RSD values of < 4.1% that are less than the predicted relative standard deviation (PRSD) of 10 to 12% at LOQ, 8% at 5 ppm and 6% at 30 ppm,

where PRSD is calculated from $0.67 \times 2C^{-0.15}$ and C is the concentration expressed as a mass fraction in the same units [61].

Accuracy [60] refers to the closeness of agreement between a measured quantity value and a true quantity value of measured. The measured amount of standards is estimated from A'_{corr} and calibration plots in Figure 4.5, where A'_{corr} is obtained from five days and each day for five runs that are performed for investigation of precision. Satisfactory accuracy of recovery as previously mentioned in Section 3.5.3. From Table 4.5, Satisfactory accuracy of 96.3-104.9% at LOQ, 95.3-103.7% at 5 ppm and 96.1-104.7% at 30 ppm, within the acceptable range of 80-110% for the concentration in a range of 0.1-10 ppm and 90-107% at 100 ppm [62].

Table 4.3 Precision in t_m of the five standard analytes at LOQ, 5 and 30 ppm.

Analytes	%RSD at LOQ						*P*-value
	Day 1	Day 2	Day 3	Day 4	Day 5	Overall	
P	0.63	0.76	1.1	1.2	0.82	0.92	0.37
PE	0.96	0.79	1.33	0.99	0.86	0.97	0.70
E	0.23	0.85	0.40	0.76	0.53	0.63	0.20
F	0.79	1.6	0.70	1.0	0.42	0.96	0.56
S	0.48	0.57	0.57	0.92	0.50	0.63	0.47
	%RSD at 5 ppm						
P	0.83	0.92	1.1	1.0	0.84	0.96	0.39
PE	0.91	0.74	0.92	0.44	0.45	0.75	0.70
E	0.78	0.75	0.65	0.26	0.39	0.59	0.52
F	0.35	0.30	0.38	0.38	0.30	0.34	0.45
S	0.33	0.20	0.15	0.28	0.22	0.25	0.36
	%RSD at 30 ppm						
P	0.83	0.92	1.1	1.0	0.84	0.47	0.58
PE	0.91	0.74	0.92	0.44	0.45	0.63	0.20
E	0.27	0.35	0.51	0.34	0.29	0.38	0.15
F	0.54	0.69	0.74	0.46	0.57	0.57	0.92
S	0.49	0.49	0.46	0.36	0.35	0.42	0.69

Data of t_m and ANOVA analysis are shown in Appendix A-1.1 to A-1.15.

Table 4.4 Precision in the measured concentration of the five standard analytes at LOQ, 5 and 30 ppm.

Analytes	%RSD at LOQ						*P*-value
	Day 1	Day 2	Day 3	Day 4	Day 5	Overall	
P	3.4	3.0	1.7	3.0	3.2	3.1	0.17
PE	4.4	1.8	1.8	4.8	1.7	3.6	0.09
E	2.7	4.1	2.6	1.5	3.1	3.2	0.12
F	2.1	3.2	2.3	1.9	1.7	2.3	0.39
S	2.7	2.3	1.4	2.1	1.2	2.3	0.06
	%RSD at 5 ppm						
P	3.5	2.1	4.0	3.6	3.3	3.5	0.21
PE	2.7	2.7	3.2	3.7	2.8	3.1	0.31
E	1.7	1.4	1.9	3.2	3.6	2.7	0.06
F	3.7	1.8	3.4	3.5	1.6	3.2	0.12
S	3.4	2.2	2.6	4.9	3.8	3.9	0.06
	%RSD at 30 ppm						
P	4.4	3.1	3.6	5.1	3.6	4.1	0.34
PE	3.4	4.0	3.1	5.0	3.1	3.8	0.32
E	4.9	3.1	3.2	3.3	4.3	3.8	0.49
F	1.4	1.9	2.4	2.6	1.5	2.1	0.17
S	3.1	2.4	3.6	3.8	4.2	3.4	0.35

Data of the measured concentration and ANOVA analysis are shown in Appendix A-2.1 to A-2.15.

Table 4.5 Satisfactory accuracy of recovery at three levels of the five standard analytes.

Analytes	Recoveries (%) at		
	LOQ	5 ppm	30 ppm
P	94.0-99.3	98.7-103.6	99.3-104.7
PE	96.5-102.2	97.9-101.7	96.1-102.9
E	95.4-100.3	98.3-103.1	98.9-104.5
F	101.4-104.1	95.3-101.7	98.5-101.4
S	93.6-97.0	96.0-102.7	96.6-101.0

4.3 Application to Real Samples

The developed CZE technique was used to quantitative determination of five adulterants in eight real samples of capsules dietary supplement for weight control as previously mentioned in Section 3.6. Qualitative identification of five analytes in real samples was perform by using a spiking technique and comparing the UV spectra of standard peaks with sample peaks at comparable t_m, at 6 to 9 minutes as shown Figure B-2 in Appendix B. In addition, the overlaid electropherograms of standards and diluted solutions of samples C1 to C4 are shown in Figure 4.6A and those of standards and samples C5 to C8 without dilution are shown in Figure 4.6B. From a spiking technique and UV spectrum comparison, samples C1 to C4 was found to adulterate only with sibutramine, while any anti-obesity drug of interest was not detected in other samples C5 to C8. From quantitative determination of sibutramine in samples C1 to C4 for triplicate runs each, results are shown in Table 4.6. A sibutramine was founded in these four samples with the concentration level of 1.7-2.7% w/w or 3.6-6.4 mg/capsule.

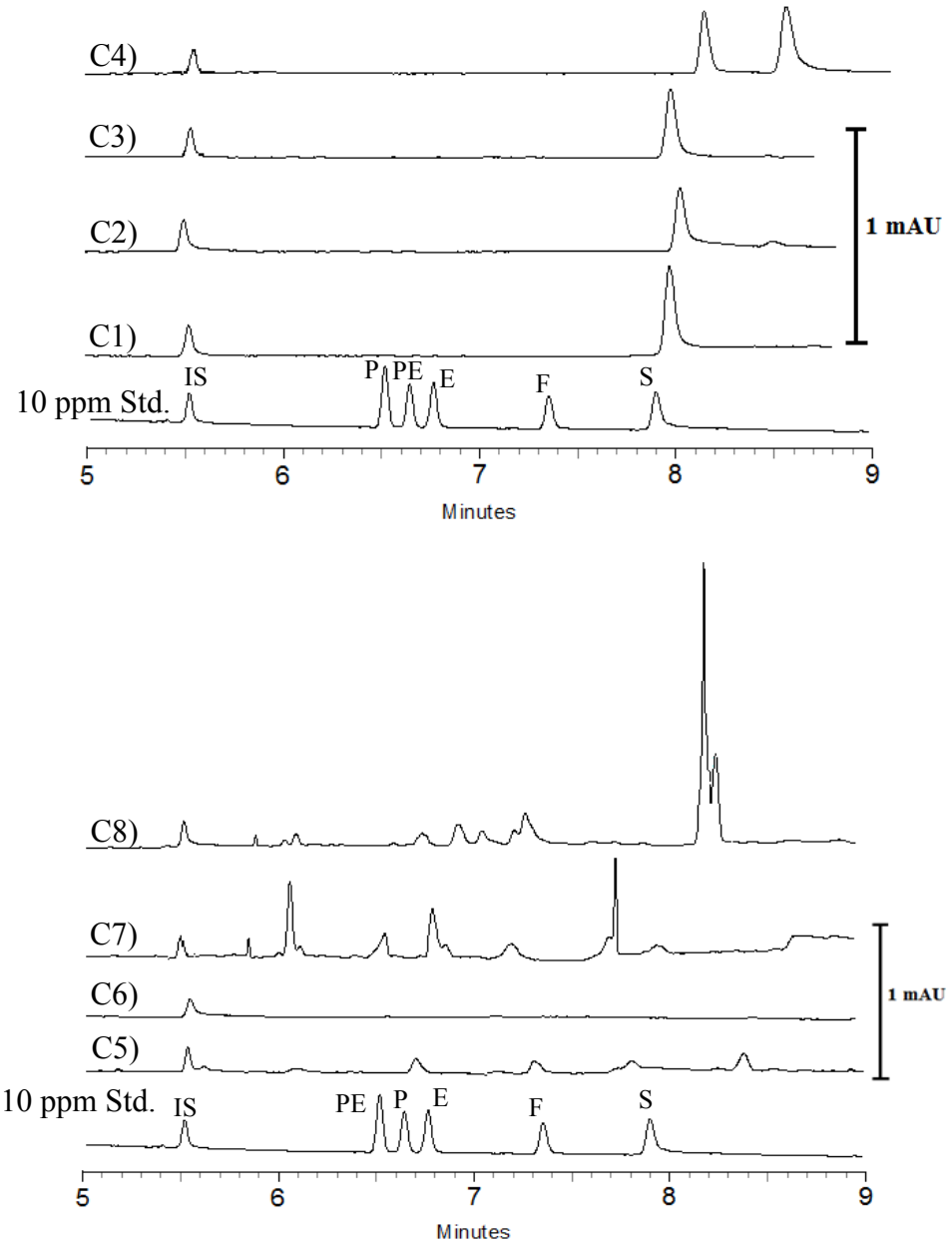


Figure 4.6 CZE electropherograms for analysis of anti-obesity drugs adulterated in dietary supplement. (A) dilution of real sample solutions found only S with standard solution, C1), C2) 150 folds-diluted sample solution, C3), C4) 100 folds-diluted sample solution (B) without dilution of real sample solutions (C5-C8). CZE conditions as shown in Figure 4.1 A.

Table 4.6 The amount of sibutramine in real samples.

Sample	Weight (mg)	Dilution factor	concentation without dilution (ppm)			% w/w				mg/capsule
			Run1	Run2	Run3	Run1	Run2	Run3	Average	
C1	248.0	150	5895	6249	5885	2.38	2.52	2.37	2.42±0.08	6.01 ± 0.21
C2	239.2	150	6783	6302	6239	2.84	2.63	2.61	2.69±0.12	6.44 ± 0.30
C3	209.4	100	4129	4278	4304	1.97	2.04	2.06	2.02±0.05	3.85 ± 0.09
C4	218.1	100	3635	3438	3764	1.67	1.58	1.73	1.66±0.08	3.61 ± 0.16

4.4 CZE-UV Separation Using a Volatile Buffer for MS Detection

This section involves a preliminary study of CZE-UV separation using a volatile buffer that will be applied to CZE-MS in the future work,

4.4.1 Choice of the Volatile Buffer in CZE-UV

Since a volatile buffer should be used for CE-MS as previously described in Section 2.8, an ammonium acetate buffer with pHs of 4, 5 and 6 was preliminary investigated in this work for simultaneous separation of analytes by CZE-UV. It should be noted that acetic acid has pK_a of 4.8, therefore the pHs of 4 to 6 are in the range of buffering capacity and provide positively charged weak base analytes (pK_a of 8.5 to 10.1) with degree of dissociation at least 0.997.

From CZE-UV electropherograms in Figure 4.7 using an ammonium acetate buffer without the organic solvent, co-migration of P and PE was found at pH 4, 5 and 6. A decrease in migration time with an increase in pHs from 4 to 6 due to an increase in μ_{eo} (Figure 4.8) caused by an enhance in q^* and ζ ($\mu_{eo} \propto \zeta$ as in Equation 2.3).

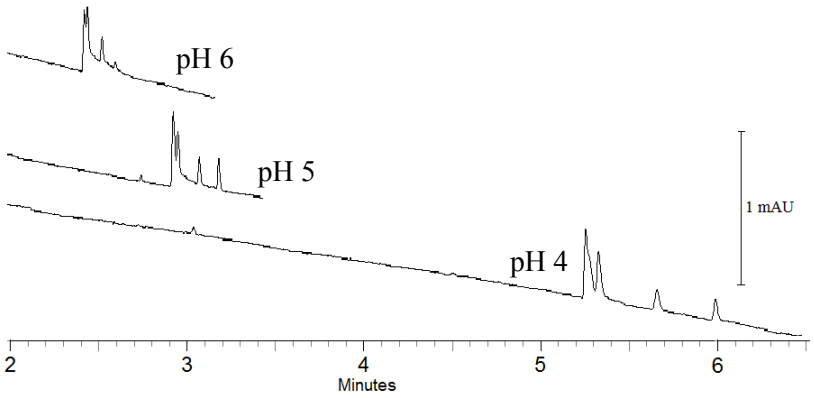


Figure 4.7 CZE electropherograms of five analytes using a 20 mM ammonium acetate buffer at pHs 4, 5 and 6 without the organic solvent.

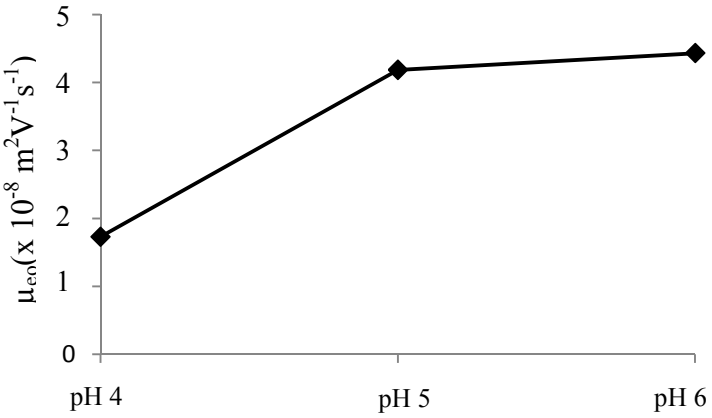


Figure 4.8 μ_{eo} in a 20 mM ammonium acetate buffer at pHs 4, 5 and 6 without organic solvent.

4.4.2 The Effect of the Type and Concentration of Organic Solvent

In the study of the effects of type and concentration of organic solvent on a change in the migrating behavior and separation of the five analytes were investigated using 0 to 50% (v/v) ACN or 0 to 70% (v/v) MeOH in ammonium acetate with pHs 4, 5 and 6, respectively, as mentioned in Section 3.7, and the CZE electropherograms are shown in Figure 4.9. The effect of organic solvents on a change in migration behavior is first discussed, such as electroosmotic mobility (μ_{eo}), electrophoretic mobility (μ) and net mobility (μ_{net}), where $\mu_{net}= \mu_{eo}+ \mu$, and all mobilities are calculated using Equations 2.6-2.8. From Figure 4.10, a slightly decrease in μ_{eo} was obtained with an increase in 0 to 50% ACN in the buffer at pHs 5 and 6 and 0 to 70% MeOH in for the buffer at

pHs 4, 5 and 6. It should be noted that $\mu_{eo} \propto \varepsilon\zeta/\eta$, as in Equations 2.3. As previously mentioned in Section 4.1.4, an increase in amount of MeOH or ACN increases the viscosity η of a solution near the capillary wall but decreases the dielectric constant ε. The ε/η ratio decreases from 90 to 61 for 100% water to 100% MeOH, but slightly increases from 90 to 110 for 100% water to 100% ACN [35]. In addition, an increase in the concentration of MeOH or ACN reduces dissociation of –SiOH to $–SiO^-$ in the buffer, resulting in a decrease in q^* and ζ. Therefore a decrease in μ_{eo} in Figure 4.10 is caused by a change in ε/η together with a decrease in ζ. However, a slight increase in μ_{eo} using a pH 4.0 the buffer with an increase in concentration of ACN may be implied that an increase in ε/η has an effect over a decrease in ζ at this pH.

From Figure 4.11(A) and 4.12 (A) using MeOH or ACN added in the buffer at pH 4.0, respectively, a decrease in μ value to minimum with an increase in the concentrations 0 to 40% MeOH or 0 to 20% ACN and then slightly increases at higher concentrations of MeOH or ACN. By assuming that α remains constant due to the pH 4 of the buffer quite far from the pK_a> 8.5 of the analytes, a change in μ is affected only by an increase in the organic solvent/water viscosity to the maximum and then a decrease in the viscosity at higher concentration of organic solvent as previously discussed in Section 4.1.4 ($\mu \propto 1/\eta$ as in Equation 2.1). It should be noted that pH 4 of the buffer is quite far from the pK_a > 8.5 of the analytes, addition of organic solvent may have little or no effect on the backward formation of fully positively charged analytes to uncharged molecules. Unlike the buffer at pH 4 ($\mu \propto 1/\eta$), the pHs 5 and 6 shows that μ decreases with an increase in the concentrations of MeOH or ACN (Figure 4.11 (B, C) and 4.12 (B, C)). This may be because pHs 5 and 6 are closed to the pK_a > 8.5 of the analytes ($\mu \propto \alpha/\eta$), and MeOH or ACN added in the buffer reduces α of the analytes, resulting in a decrease in μ at higher the concentration of MeOH or ACN.

From preliminary study of CZE-UV separation using a volatile buffer, it can be suggested that a pH 4.0 ammonium acetate buffer containing 30 to 50% ACN or 40 to 70% MeOH be used in order to achieve base separation of five analytes. However,

CZE-MS in our laboratory is not available to operate, the CZE-MS on separation and detection of five analytes has been not performed.

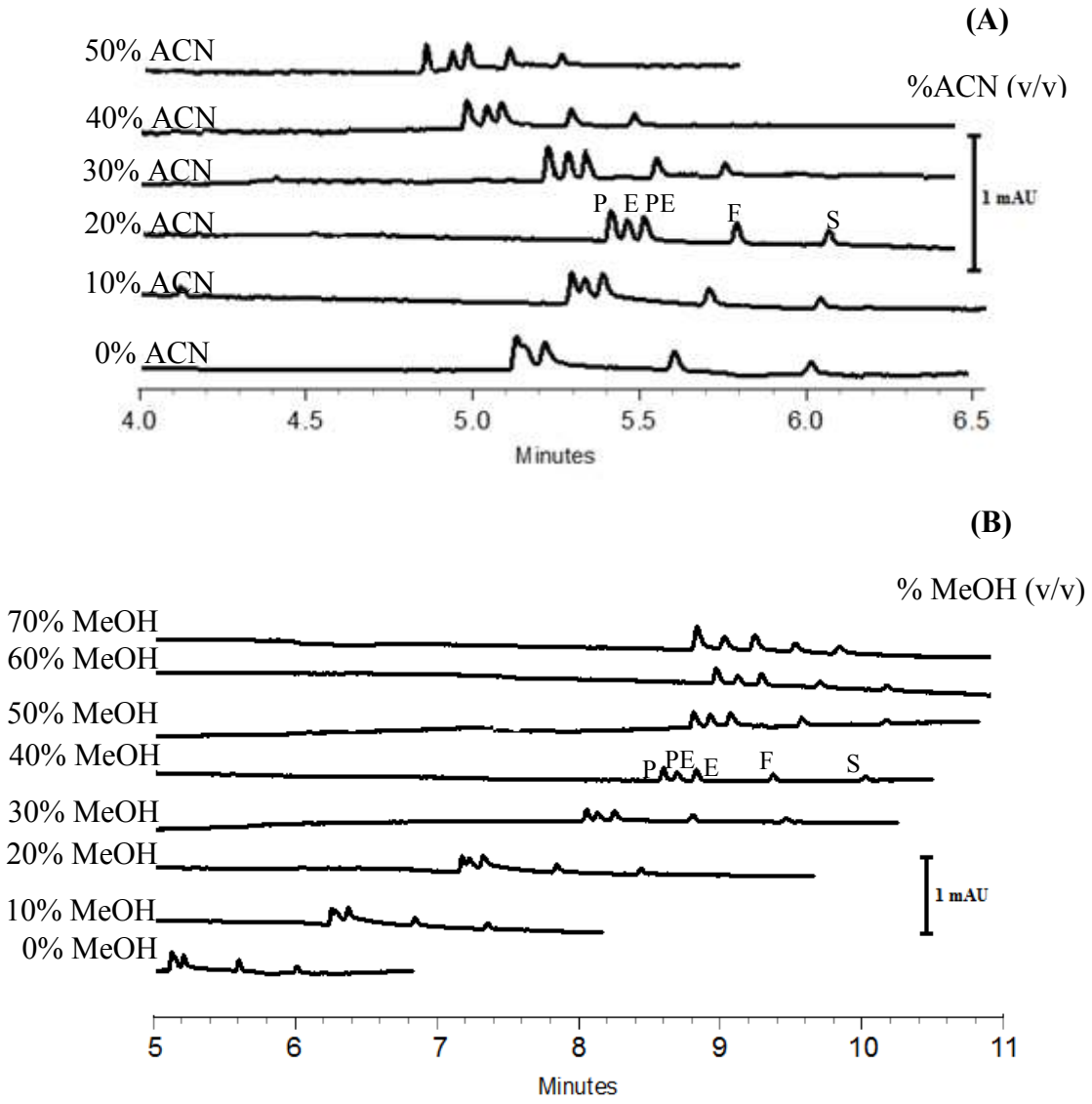


Figure 4.9 CZE electropherograms of five analytes using organic solvents, (A) 0-50% ACN, (B) 0-70% MeOH, added into a 20 mM ammonium acetate buffer at pH 4. CE conditions: uncoated fused silica capillary 50 µm i.d. × 60.2 cm (50 cm to detector), temperature 25 ^{o}C, voltage of +25 kV, 0.5 psi pressure injection for 10 s and UV detection at 214 nm.

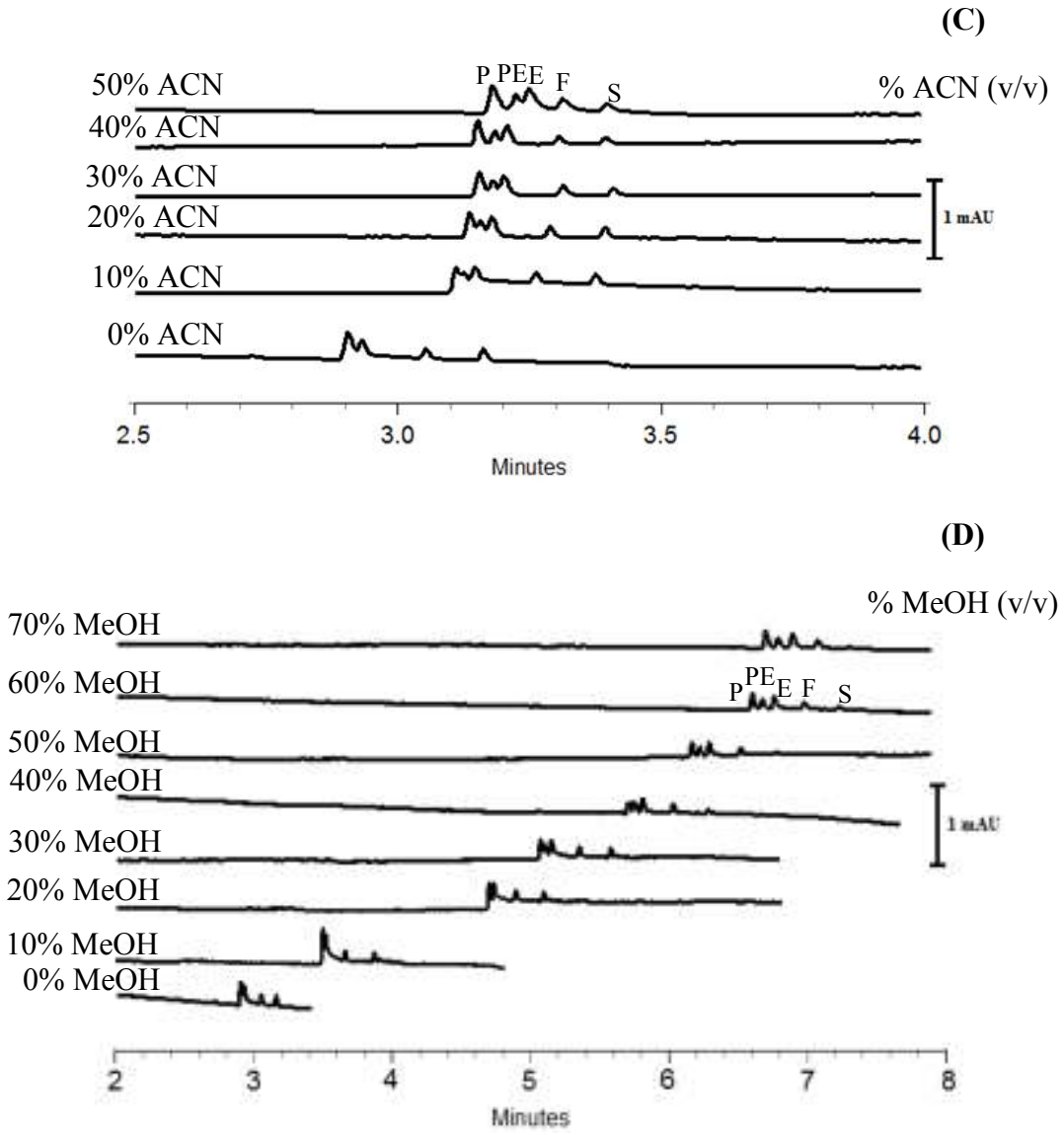


Figure 4.9 CZE electropherograms of five analytes using organic solvents, (C) 0-50% ACN, (D) 0-70% MeOH, added into a 20 mM ammonium acetate buffer at pH 5. CE conditions: uncoated fused silica capillary 50 µm i.d. × 60.2 cm (50 cm to detector), temperature 25 ^{o}C, voltage of +25 kV, 0.5 psi pressure injection for 10 s and UV detection at 214 nm.

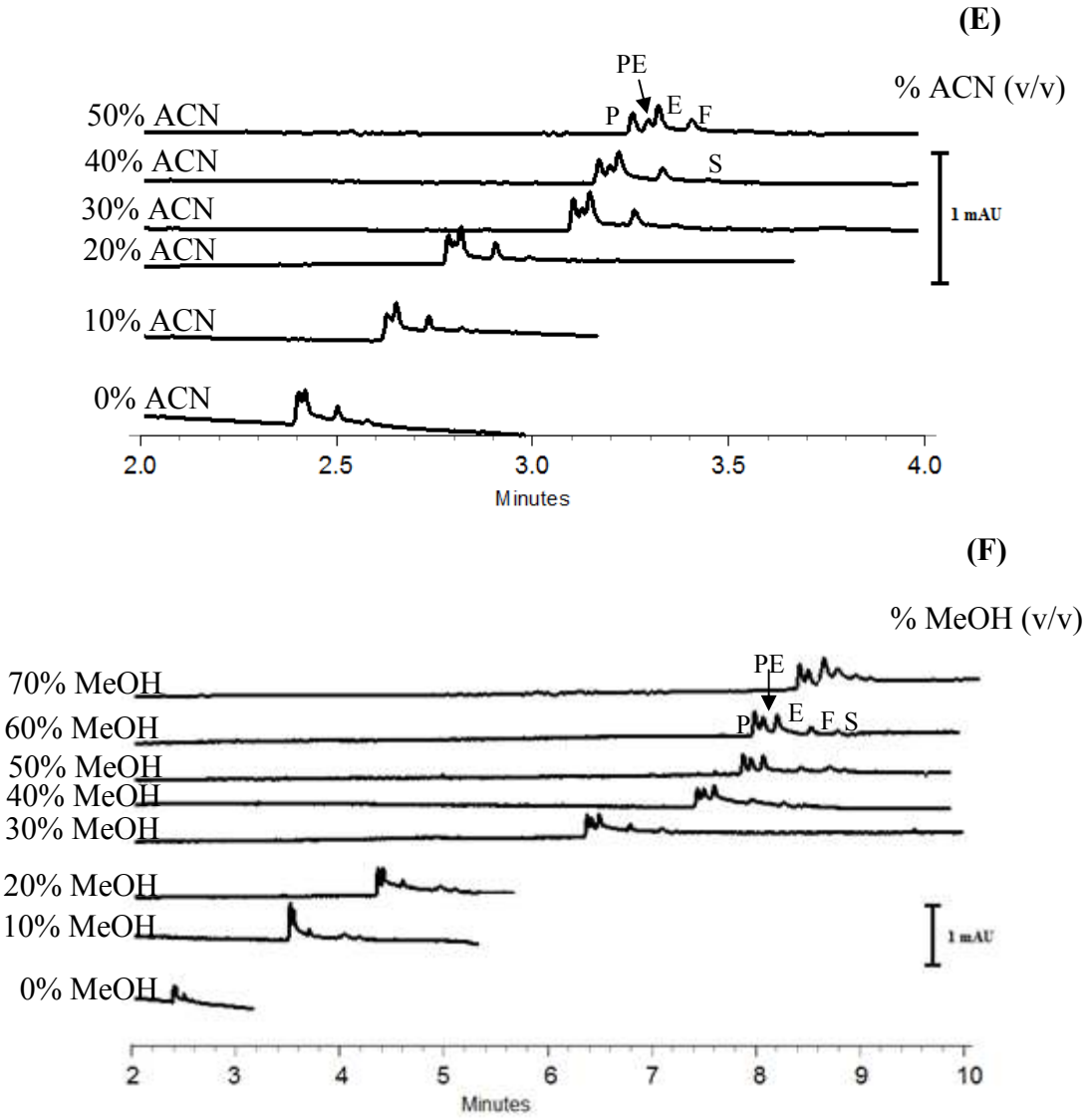


Figure 4.9 CZE electropherograms of five analytes using organic solvents, (E) 0-50% ACN, (F) 0-70% MeOH, added into a 20 mM ammonium acetate buffer at pH 6. CE conditions: uncoated fused silica capillary 50 µm i.d. × 60.2 cm (50 cm to detector), temperature 25 ^{o}C, voltage of +25 kV, 0.5 psi pressure injection for 10 s and UV detection at 214 nm.

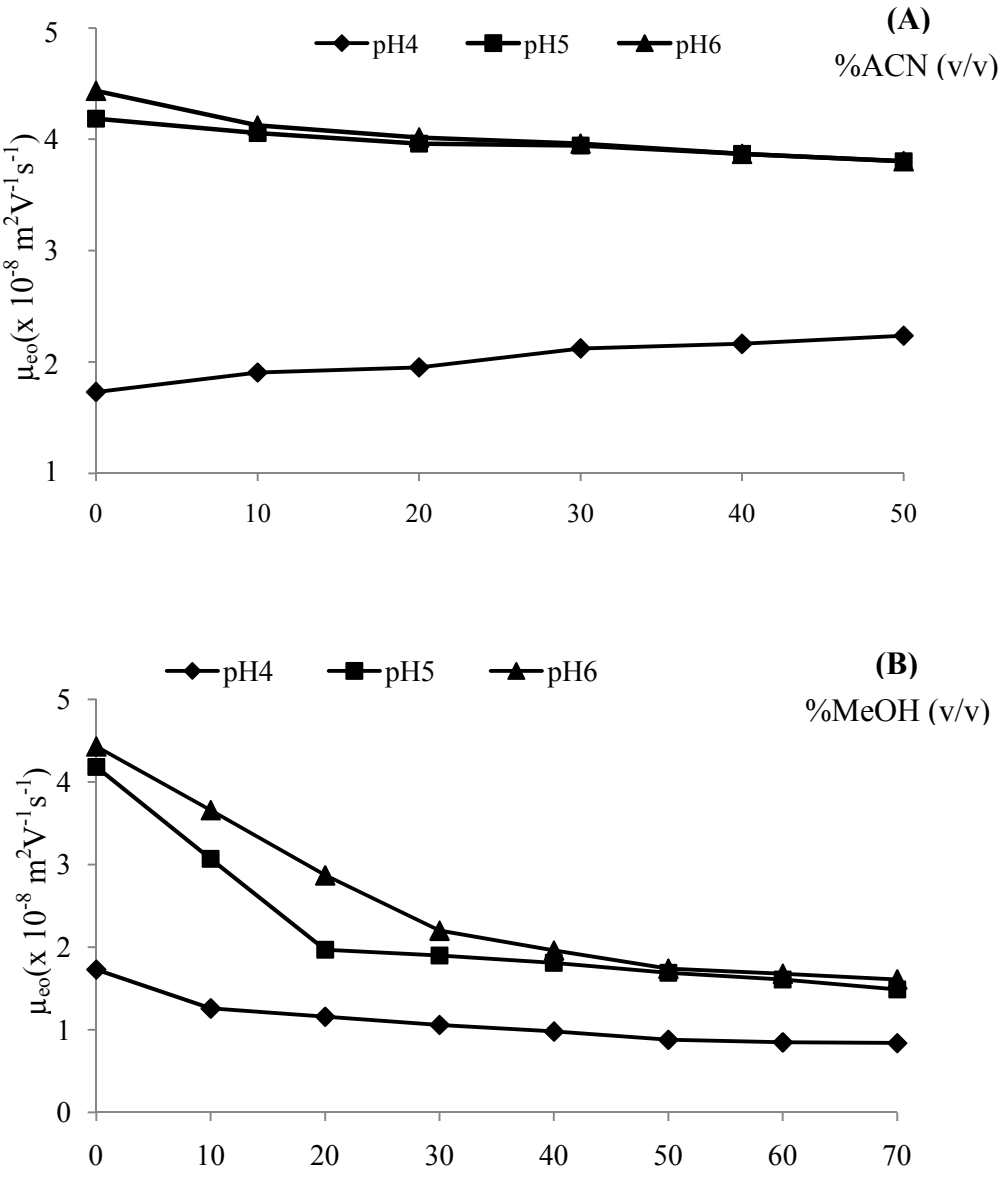


Figure 4.10 μ_{eo} in a 20 mM ammonium acetate buffer at pHs 4, 5 and 6 containing (A) 0-50% ACN and (B) 0-70% MeOH.

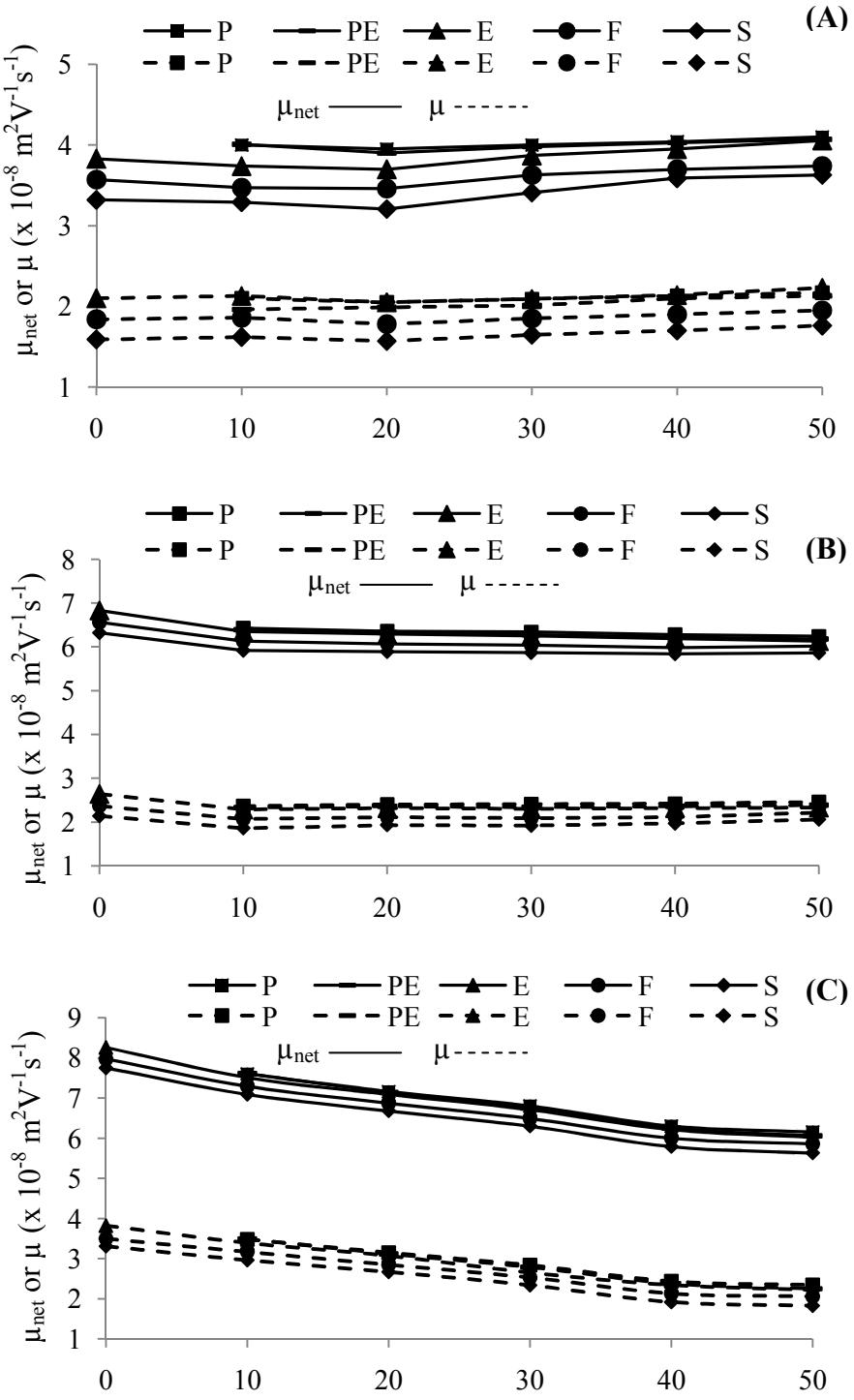


Figure 4.11 μ and μ_{net} in a 20 mM ammonium acetate buffer containing 0-50% ACN at (A) pH 4, (B) pH 5 and (C) pH 6.

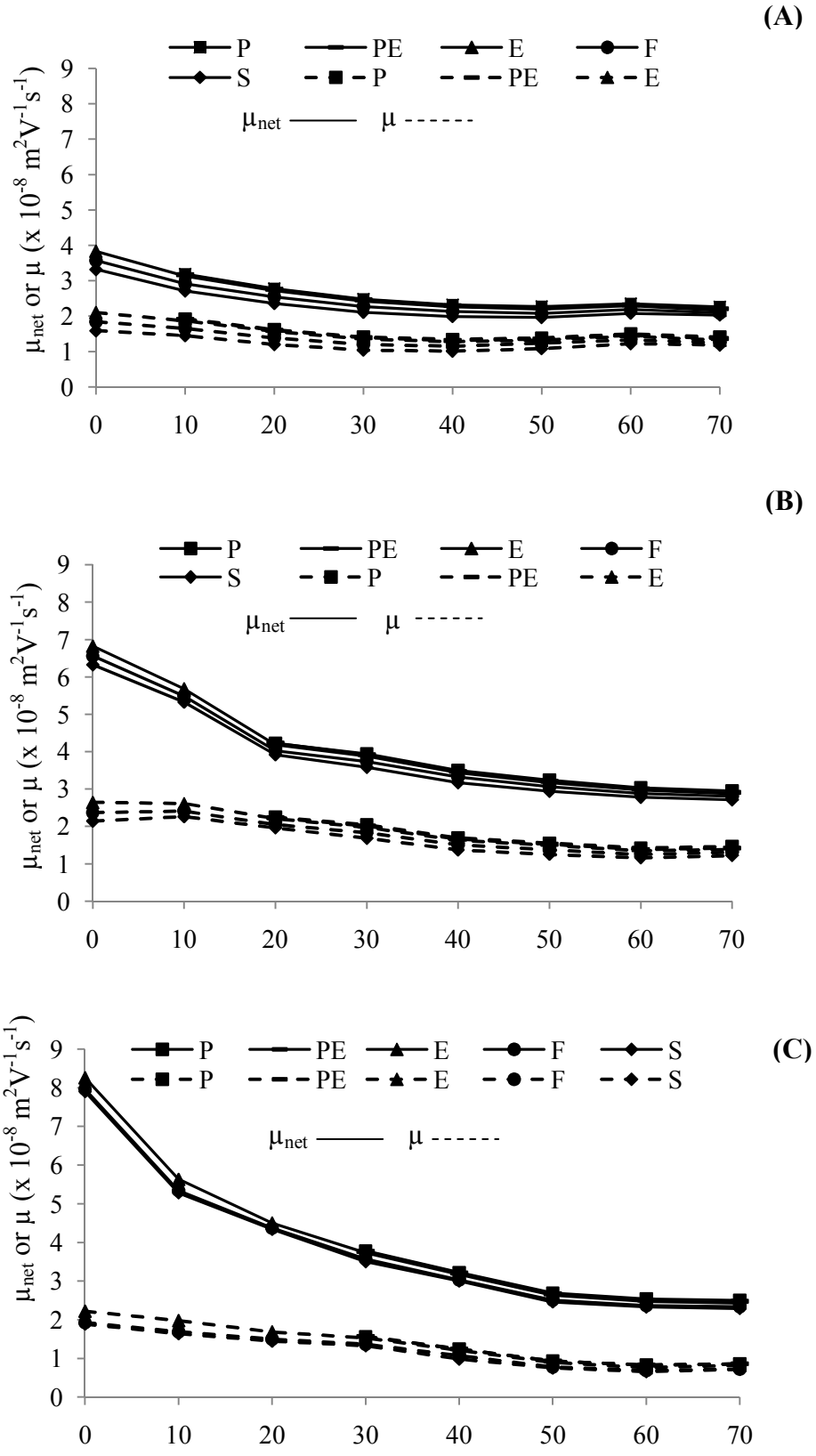
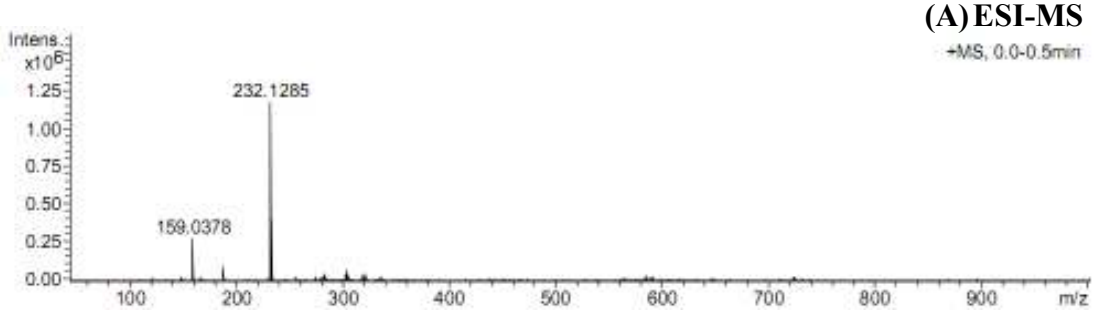


Figure 4.12 μ and μ_{net} in a 20 mM ammonium acetate buffer containing 0-70% MeOH at (A) pH 4, (B) pH 5 and (C) pH 6.

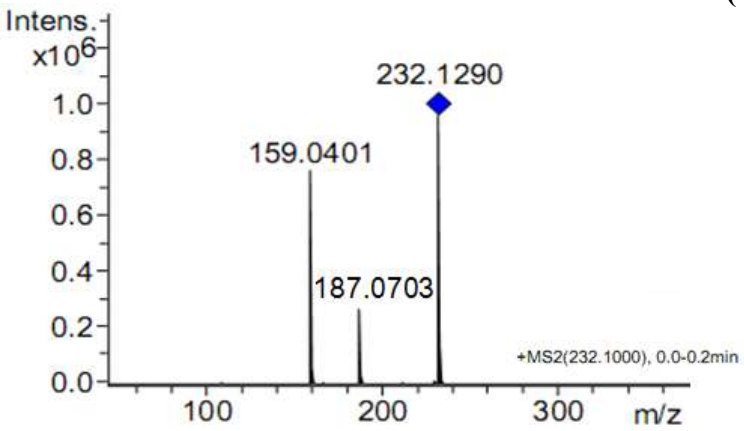
4.5 Mass Spectra of Five Analytes Studied by ESI-MS and ESI-MS/MS

Since CZE-MS is not available to operate, ESI-MS and ESI-MS/MS were used to study mass spectra of five analytes in order to obtain structural information of the analytes. Each analyte was separately injected directly to ESI-MS or ESI-MS/MS using a syringe pump, positive ESI mode, and a quandrupole as a mass analyzer for ESI-MS and quandrupole/time-of-flight as mass analyzers for ESI-MS/MS. More details of ESI-MS and ESI-MS/MS conditions are given in Section 3.8. Using ESI-MS, protonated molecules $[M+H]^+$ are 232.13 m/z for fenfluramine (Figure 4.13A), 166.12 m/z for ephedrine (Figure 4.14A) and pseudoephedrine (Figure 4.15A), 150.12 m/z for phentermine (Figure 4.16A) and 280.18 m/z for sibutramine (Figure 4.17A). In ESI-MS/MS was used study mass pathway of each analytes from different fragment ions or product ions which generated from as precursor ion $[M+H]^+$which their product ion mass spectra and mass pathway of each analytes as shown in Figure 4.13B-4.17B and 4.13C-4.17C, respectively. The product ions of each analyte such as 187.07 and 159.04 m/z for fenfluramine (Figure 4.13B), 148.11, 133.09 and 117.07 m/z for ephedrine (Figure 4.14B) and pseudoephedrine (Figure 4.15B), 133.10, 105.07, and 91.05 m/z for phentermine (Figure 4.16B) and 179.06, 153.04, 139.03 and 125.01 m/z for sibutramine (Figure 4.17B). This results provide high sensitivity, fast analysis time andcan be use confirm mass and supported structural information for an unambiguous identification in the determination of analytes.

(A) ESI-MS



(B) ESI-MS/MS



(C) fragmentation pathways

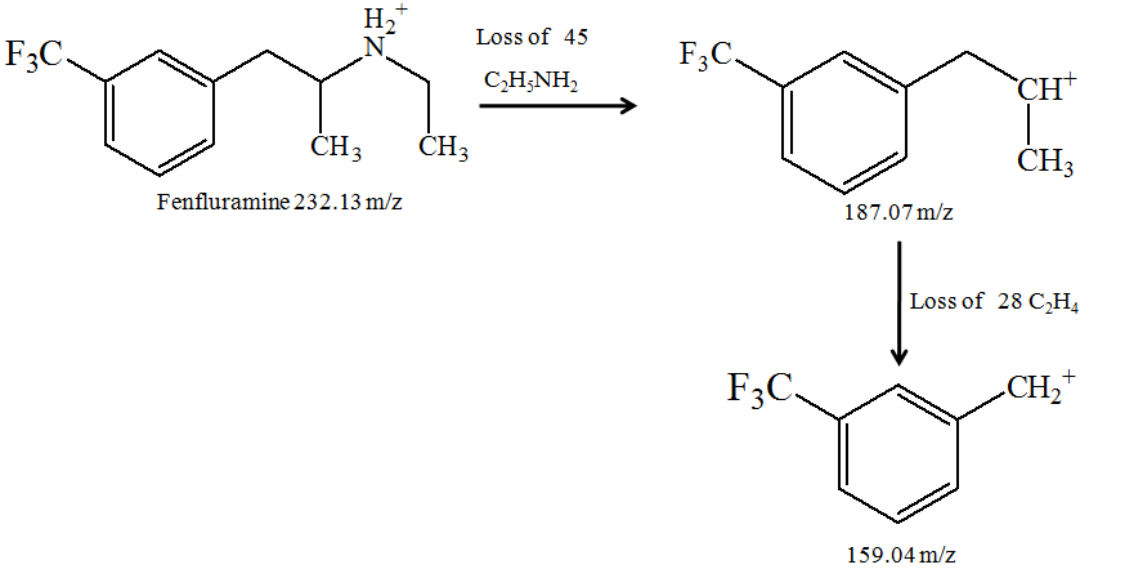


Figure 4.13 Mass spectra and fragmentation pathway of fenfluramine.

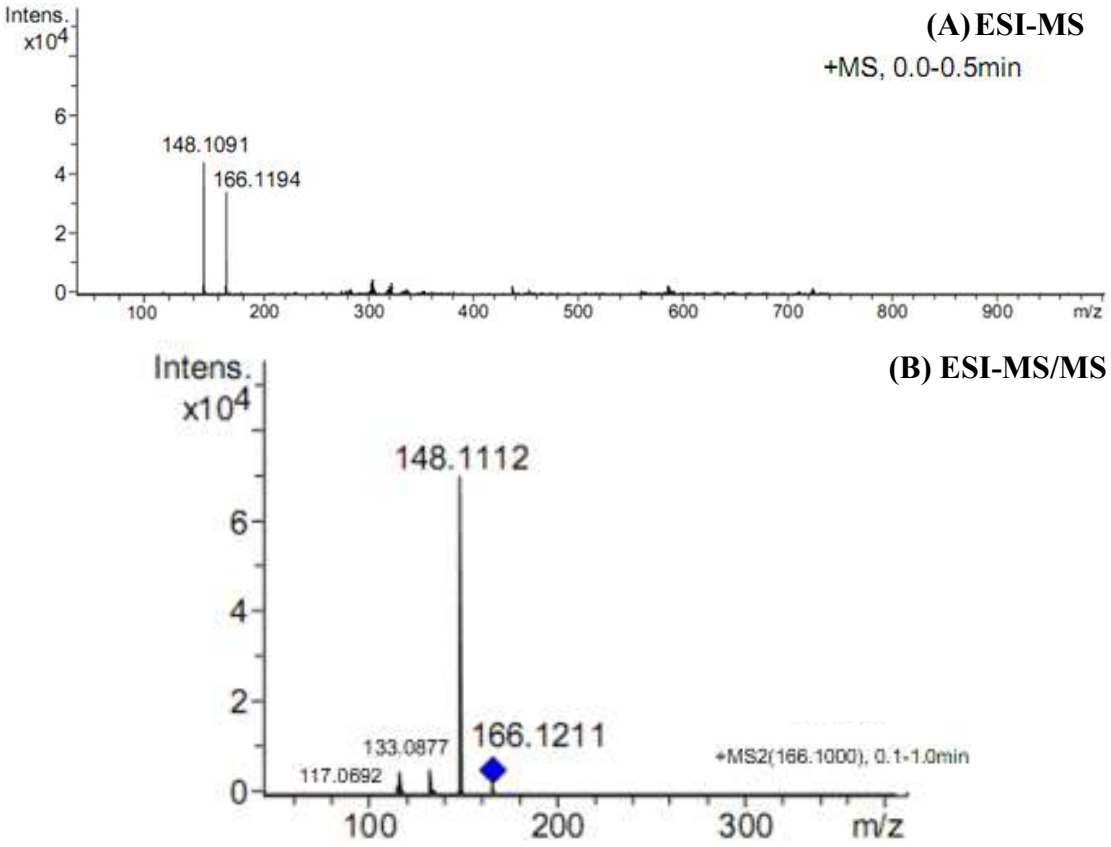
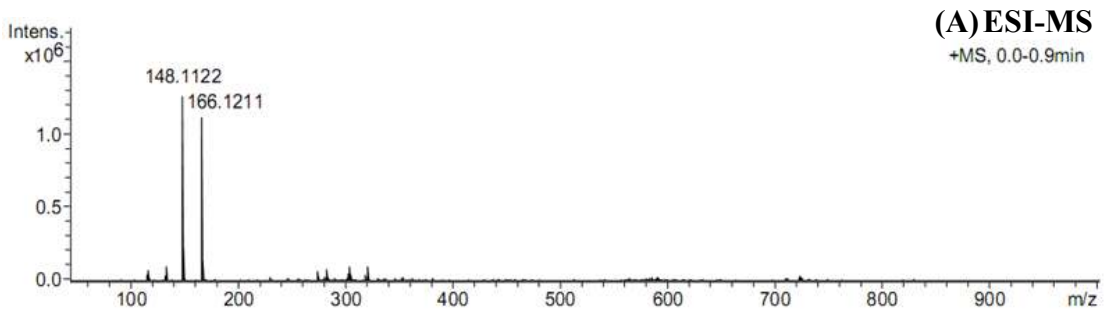
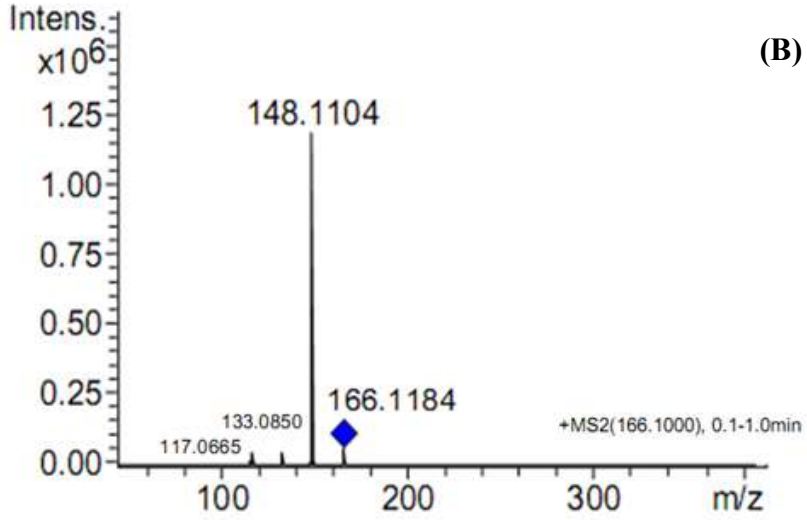


Figure 4.14 Mass spectra and fragmentation pathway of ephedrine.





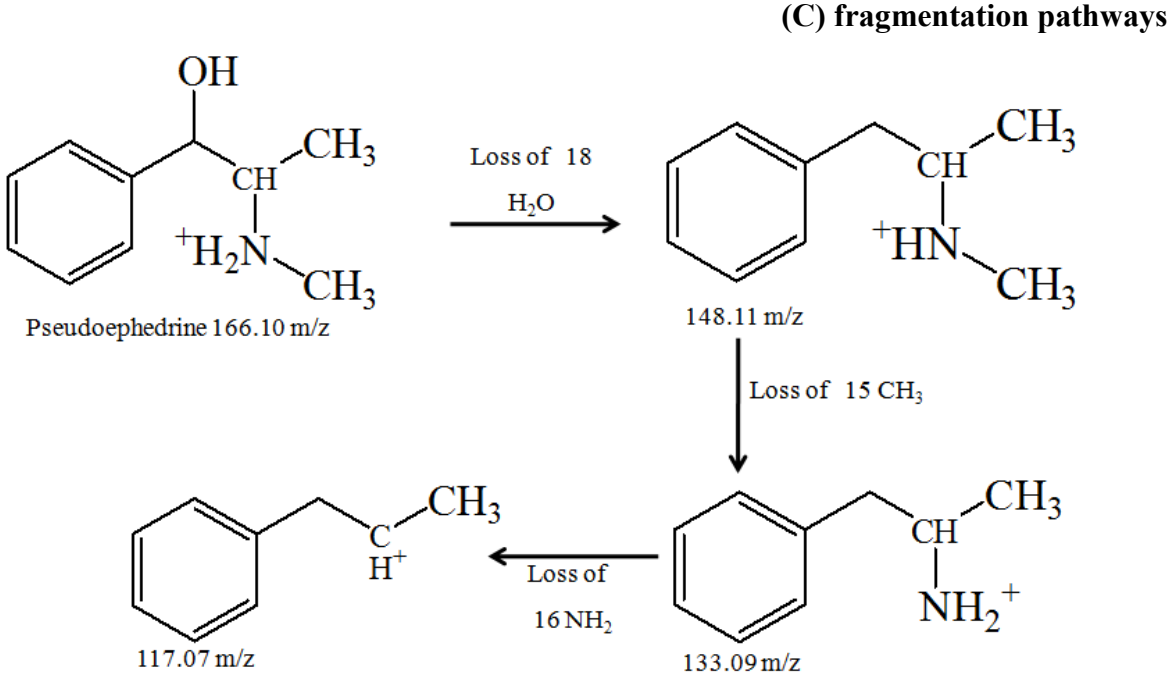


Figure 4.15 Mass spectra and fragmentation pathway of pseudoephedrine.

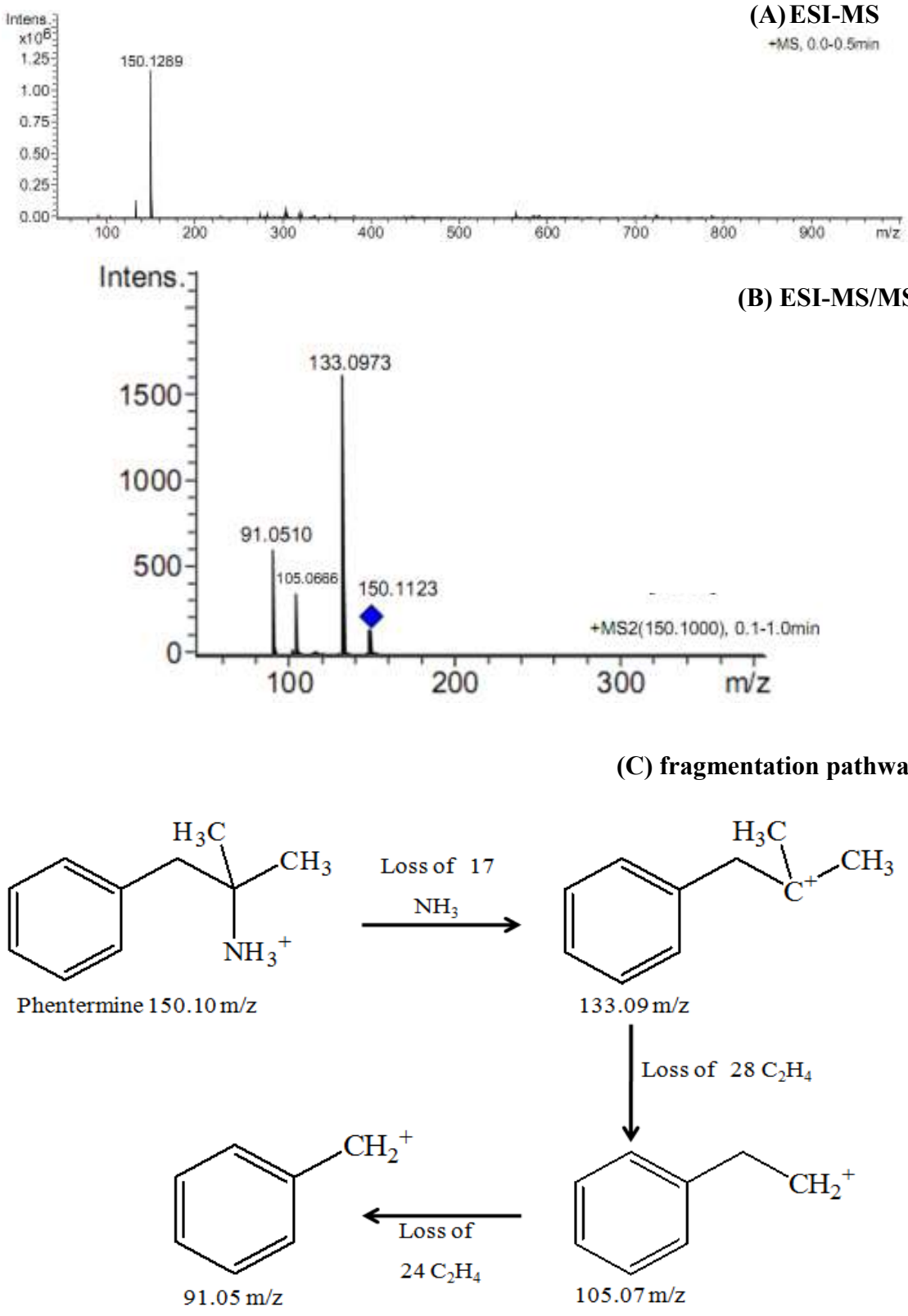


Figure 4.16 Mass spectra and fragmentation pathway of phentermine.

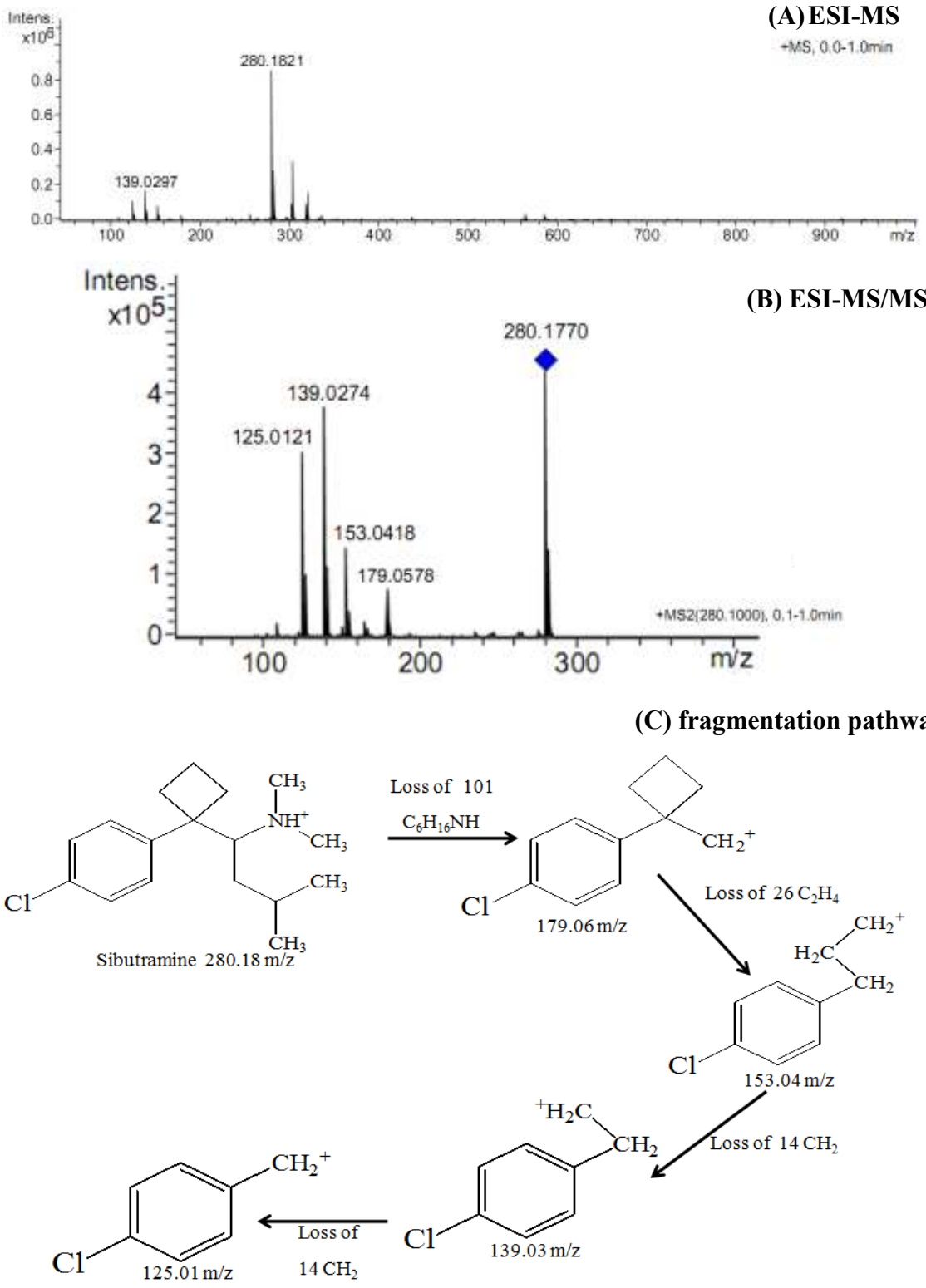


Figure 4.17 Mass spectra and fragmentation pathway of sibutramine.

CHAPTER V
CONCLUSION

Capillary zone electrophoresis (CZE) was optimized and validated for simultaneous separation and quantitative determination of five anti-obesity drugs adulterated in dietary supplement capsules including fenfluramine (F), ephedrine (E), pseudoephedrine (PE), phentermine (P) and sibutramine (S). Preliminary study of CZE-UV separation using a volatile buffer for CZE-MS was also investigated for these five analytes. In addition, ESI-MS and ESI-MS/MS were used to obtain product ion mass spectra of the five analytes.

In the initial work, CZE separation was performed using uncoated fused silica capillary with 50 μm i.d. × 60.2 cm (50 cm to detector), the separation temperature of 25 ^{o}C, voltage of +25 kV, 0.5 psi pressure injection for 10 s, and photo diode array-UV detection by scanning in a range of 200-400 nm and monitoring at 214 nm. A pH 2.5 100 mM tris-phosphate buffer was chosen in order that five weak base analytes of interest, with their pK_a in a range of 8-10, carry a fully positive charge (pH < pK_a at least 2 units). Phosphate anions do not disturb the measurement with UV detector (range 200-300 nm). A tris-H^+ co-ion was used to obtain electrophoretic mobility matching between the co-ion and analyte ions in order to reduce peak distortion caused by electromigration dispersion. Adding organic solvent in the buffer affects a change in electrophoretic mobilities of the analytes, and therefore, may lead to improve simultaneous separation of the several analytes. In comparison between 0 to 40% ACN and 0 to 70% MeOH added into the buffer, ACN provided less migration time (t_m) and better separation of five test analytes. In order to compromise between fast analysis time, achieve base line resolution (R_s > 2.0 for quantitative analysis), 20% ACN in a 100 mM phosphate buffer at pH 2.5 was chosen for validation of the method and application to real samples.

The developed CZE method was then validated for quantitative determination of five drugs. Instrumental LOD and LOQ were found in a range of 0.1 to 0.2 ppm and 0.45

to 1.0 ppm, respectively. The calibration plot were established by plotting A'_{corr} against the concentrations of each standard at seven levels in a range of LOQ to 50 ppm, where A'_{corr} is the ratio of corrected peak area, peak area divided by migration time, of the standard to that of the internal standard. Highly linear relationship was obtained with coefficient of determination (r^2) ranging from 0.9990 to 0.9998. The accuracy and precision for quantitative analysis were assessed at three concentrations levels of LOQ, 5 and 30 ppm. Intraday precision in t_m and the measured concentration was evaluated from five consecutive runs, while interday precision from five consecutive days and each day for five runs was performed. For overall five analytes at these three concentrations levels above mentioned, non-significant difference in relative standard deviation (RSD) was obtained between intraday and interday precision with RSD < 1% for t_m and < 4.1% for the measured concentration, respectively. Satisfactory accuracy was also obtained with recoveries of 96.3-104.9% at LOQ, 95.3-103.7% at 5 ppm and 96.1-104.7% at 30 ppm, within the acceptable range of 80-110% for the concentration in a range of 0.1-10 ppm and 90-107% at 100 ppm. Therefore, satisfactory validation parameters, such as LOD, LOQ, linearity, accuracy and precision, were obtained. This CZE method was used for quantitative determination of five anti-obesity drugs in eight samples of capsule dietary supplements from different suppliers. Using a spiking technique and comparing the UV spectra of standard peaks with the peaks of the sample at comparable t_m, only sibutramine was found in four out of these eight samples with the amount levels of 1.6-2.7% w/w or 3.6-6.4 mg/capsule. Therefore, CZE can be used as an alternative method for analysis of anti-obesity drugs adulterated in dietary supplement.

Preliminary study of CZE-UV separation using a volatile buffer for CZE-MS was also investigated for these five analytes. With other CE conditions similar to abovementioned, a 20 mM ammonium acetate buffer at pHs 4, 5 and 6 and with 0-50% ACN or 0-70% MeOH (v/v). In order to achieve base separation of five analytes, a pH 4.0 ammonium acetate buffer containing 30 to 50% ACN or 40 to 70% MeOH should be used. However, CZE-MS in our laboratory is not available to operate, the CZE-MS on separation and detection of five analytes has been not performed.

ESI-MS and ESI-MS/MS were used to study product ions mass spectra of five analytes in order to obtain structural information of the analytes. This information may be used to confirm the analytes detected in the real sample for the future work.

The advantages of developed CZE method for determination of five contraband anti-obesity drugs adulterated in supplements for weight control include fast analysis time within 10 min, good resolution and simpler sample preparation, LOD down to less than 1 ppm, high linearity and satisfactory accuracy and precision. CZE method can be used coupling with MS for confirming and studying structural information of these drugs. Therefore, this developed CZE method can be used as an alternative and may be applied to other dietary supplements (table, tea or coffee) for determination of anti-obesity drug in dietary supplements.

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APPENDICES

APPENDIX A

Table A-1 Data of t_m and ANOVA satistical analysis of five analytes at LOQ, 5.0 and 30.0 ppm

A-1.1 Phentermine at 0.45 ppm

	t_m (min)					Anova: Single Factor SUMMARY				
						Groups	*Count*	*Sum*	*Average*	*Variance*
Day 1	6.635	6.686	6.642	6.726	6.719	Day 1	5	33.41	6.682	0.0018
Day 2	6.629	6.675	6.703	6.769	6.686	Day 2	5	33.46	6.692	0.0026
Day 3	6.635	6.643	6.709	6.739	6.809	Day 3	5	33.54	6.707	0.0052
Day 4	6.719	6.636	6.618	6.773	6.789	Day 4	5	33.54	6.707	0.0060
Day 5	6.769	6.685	6.716	6.818	6.798	Day 5	5	33.79	6.757	0.0031

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.017	4	0.0042	1.12	0.37	2.87
Within Groups	0.075	20	0.0037			
Total	0.092	24				

A-1.2 Phentermine at 5.0 ppm

	t_m (min)					Anova: Single Factor SUMMARY				
						Groups	*Count*	*Sum*	*Average*	*Variance*
Day 1	6.675	6.613	6.668	6.722	6.759	Day 1	5	33.44	6.687	0.0031
Day 2	6.683	6.703	6.656	6.773	6.803	Day 2	5	33.62	6.724	0.0038
Day 3	6.658	6.668	6.697	6.817	6.796	Day 3	5	33.64	6.727	0.0055
Day 4	6.654	6.636	6.765	6.795	6.717	Day 4	5	33.57	6.713	0.0047
Day 5	6.678	6.783	6.764	6.830	6.794	Day 5	5	33.85	6.770	0.0032

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.018	4	0.0044495	1.09	0.39	2.87
Within Groups	0.081	20	0.0040739			
Total	0.099	24				

A-1.3 Phentermine at 30.0 ppm

	t_m (min)					Anova: Single Factor SUMMARY				
						Groups	*Count*	*Sum*	*Average*	*Variance*
Day 1	6.683	6.697	6.702	6.732	6.758	**Day 1**	5	33.57	6.714	0.00091
Day 2	6.676	6.690	6.719	6.759	6.786	**Day 2**	5	33.63	6.726	0.0021
Day 3	6.702	6.687	6.726	6.759	6.743	**Day 3**	5	33.62	6.723	0.00086
Day 4	6.671	6.728	6.711	6.732	6.754	**Day 4**	5	33.60	6.719	0.00096
Day 5	6.727	6.752	6.774	6.730	6.750	**Day 5**	5	33.73	6.747	0.00038

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.0031	4	0.0008	0.74	0.58	2.87
Within Groups	0.021	20	0.0010			
Total	0.024	24				

A-1.4 Pseudoephedrine at 0.50 ppm

	t_m (min)					Anova: Single Factor SUMMARY				
						Groups	*Count*	*Sum*	*Average*	*Variance*
Day 1	6.732	6.757	6.782	6.895	6.837	**Day 1**	5	34.00	6.801	0.0043
Day 2	6.736	6.787	6.829	6.856	6.737	**Day 2**	5	33.95	6.789	0.0029
Day 3	6.735	6.739	6.859	6.872	6.944	**Day 3**	5	34.15	6.830	0.0082
Day 4	6.840	6.752	6.792	6.821	6.933	**Day 4**	5	34.14	6.828	0.0046
Day 5	6.837	6.779	6.815	6.937	6.856	**Day 5**	5	34.22	6.845	0.0035

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.0104638	4	0.003	0.56	0.70	2.87
Within Groups	0.094	20	0.005			
Total	0.1043598	24				

A-1.5 Pseudoephedrine at 5.0 ppm

	t_m (min)				
Day 1	6.711	6.743	6.779	6.814	6.869
Day 2	6.717	6.784	6.796	6.843	6.836
Day 3	6.737	6.785	6.867	6.892	6.841
Day 4	6.789	6.801	6.844	6.782	6.845
Day 5	6.804	6.775	6.811	6.859	6.82

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Day 1	5	33.92	6.783	0.0038
Day 2	5	33.98	6.795	0.0025
Day 3	5	34.12	6.824	0.0040
Day 4	5	34.06	6.812	0.00092
Day 5	5	34.07	6.814	0.00092

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.005	4	0.001	0.55	0.70	2.87
Within Groups	0.049	20	0.002			
Total	0.054	24				

A-1.6 Pseudoephedrine at 30.0 ppm

	t_m (min)				
Day 1	6.818	6.837	6.815	6.854	6.828
Day 2	6.731	6.753	6.836	6.853	6.848
Day 3	6.785	6.811	6.856	6.839	6.827
Day 4	6.748	6.859	6.737	6.825	6.802
Day 5	6.854	6.892	6.837	6.884	6.803

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Day 1	5	34.152	6.830	0.00025
Day 2	5	34.021	6.804	0.0033
Day 3	5	34.118	6.824	0.00074
Day 4	5	33.971	6.794	0.0027
Day 5	5	34.27	6.854	0.0013

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.011	4	0.003	1.65	0.20	2.87
Within Groups	0.033	20	0.002			
Total	0.044	24				

A-1.7 Ephedrine at 0.50 ppm

	t_m (min)					Anova: Single Factor SUMMARY				
						Groups	*Count*	*Sum*	*Average*	*Variance*
Day 1	6.818	6.837	6.815	6.854	6.828	**Day 1**	5	34.15	6.830	0.00025
Day 2	6.731	6.753	6.836	6.853	6.848	**Day 2**	5	34.02	6.804	0.0033
Day 3	6.785	6.811	6.856	6.839	6.827	**Day 3**	5	34.12	6.824	0.00074
Day 4	6.748	6.859	6.737	6.825	6.802	**Day 4**	5	33.97	6.794	0.0027
Day 5	6.854	6.892	6.837	6.884	6.803	**Day 5**	5	34.27	6.854	0.0013

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.011	4	0.0027	1.65	0.20	2.87
Within Groups	0.033	20	0.0017			
Total	0.044	24				

A-1.8 Ephedrine at 5.0 ppm

	t_m (min)					Anova: Single Factor SUMMARY				
						Groups	*Count*	*Sum*	*Average*	*Variance*
Day 1	6.828	6.857	6.885	6.959	6.932	**Day 1**	5	34.46	6.892	0.0029
Day 2	6.821	6.857	6.896	6.917	6.953	**Day 2**	5	34.44	6.889	0.0026
Day 3	6.875	6.823	6.913	6.899	6.943	**Day 3**	5	34.45	6.891	0.0020
Day 4	6.917	6.933	6.925	6.958	6.911	**Day 4**	5	34.64	6.929	0.00034
Day 5	6.879	6.901	6.949	6.893	6.92	**Day 5**	5	34.54	6.908	0.00074

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.006	4	0.0014	0.84	0.52	2.87
Within Groups	0.034	20	0.0017			
Total	0.040	24				

A-1.9 Ephedrine at 30.0 ppm

	t_m (min)					Anova: Single Factor SUMMARY				
						Groups	*Count*	*Sum*	*Average*	*Variance*
Day 1	6.906	6.928	6.914	6.950	6.942	**Day 1**	5	34.64	6.928	0.00034
Day 2	6.880	6.915	6.903	6.921	6.945	**Day 2**	5	34.56	6.913	0.00057
Day 3	6.852	6.892	6.910	6.924	6.946	**Day 3**	5	34.52	6.905	0.0013
Day 4	6.898	6.932	6.954	6.918	6.951	**Day 4**	5	34.65	6.931	0.00055
Day 5	6.973	6.931	6.925	6.958	6.934	**Day 5**	5	34.72	6.944	0.00042

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.005	4	0.001	1.92	0.15	2.87
Within Groups	0.013	20	0.0006			
Total	0.017	24				

A-1.10 Fentermine at 0.55 ppm

	t_m (min)					Anova: Single Factor SUMMARY				
						Groups	*Count*	*Sum*	*Average*	*Variance*
Day 1	7.452	7.496	7.423	7.569	7.536	**Day 1**	5	37.48	7.495	0.0035
Day 2	7.488	7.542	7.254	7.519	7.459	**Day 2**	5	37.26	7.452	0.013
Day 3	7.469	7.455	7.484	7.528	7.584	**Day 3**	5	37.52	7.504	0.0028
Day 4	7.437	7.462	7.546	7.589	7.617	**Day 4**	5	37.65	7.530	0.0061
Day 5	7.484	7.524	7.556	7.507	7.478	**Day 5**	5	37.55	7.510	0.0010

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.02	4	0.004	0.77	0.56	2.87
Within Groups	0.11	20	0.005			
Total	0.12	24				

A-1.11 Fentermine at 5.0 ppm

	t_m (min)					Anova: Single Factor SUMMARY				
						Groups	*Count*	*Sum*	*Average*	*Variance*
Day 1	7.485	7.505	7.554	7.535	7.517	**Day 1**	5	37.60	7.519	0.00071
Day 2	7.517	7.495	7.520	7.511	7.556	**Day 2**	5	37.60	7.520	0.00050
Day 3	7.480	7.525	7.538	7.513	7.557	**Day 3**	5	37.61	7.523	0.00083
Day 4	7.504	7.553	7.526	7.571	7.511	**Day 4**	5	37.67	7.533	0.00080
Day 5	7.515	7.554	7.535	7.576	7.550	**Day 5**	5	37.73	7.546	0.00052

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.003	4	0.0007	0.97	0.45	2.87
Within Groups	0.013	20	0.0007			
Total	0.016	24				

A-1.12 Fentermine at 30.0 ppm

	t_m (min)					Anova: Single Factor SUMMARY				
						Groups	*Count*	*Sum*	*Average*	*Variance*
Day 1	7.487	7.507	7.558	7.566	7.581	**Day 1**	5	37.70	7.540	0.0016
Day 2	7.456	7.494	7.570	7.534	7.579	**Day 2**	5	37.63	7.527	0.0027
Day 3	7.487	7.446	7.536	7.586	7.554	**Day 3**	5	37.61	7.522	0.0031
Day 4	7.496	7.532	7.589	7.541	7.564	**Day 4**	5	37.72	7.544	0.0012
Day 5	7.486	7.505	7.546	7.570	7.588	**Day 5**	5	37.70	7.539	0.0018

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.002	4	0.0005	0.22	0.92	2.87
Within Groups	0.042	20	0.0021			
Total	0.044	24				

A-1.13 Sibutramine at 1.00 ppm

	t_m (min)					Anova: Single Factor SUMMARY				
						Groups	*Count*	*Sum*	*Average*	*Variance*
Day 1	7.773	7.839	7.804	7.868	7.849	**Day 1**	5	39.13	7.827	0.0014
Day 2	7.779	7.855	7.816	7.792	7.735	**Day 2**	5	38.98	7.795	0.0020
Day 3	7.781	7.791	7.804	7.847	7.887	**Day 3**	5	39.11	7.822	0.0020
Day 4	7.763	7.799	7.868	7.910	7.934	**Day 4**	5	39.27	7.855	0.0053
Day 5	7.785	7.846	7.872	7.809	7.782	**Day 5**	5	39.09	7.819	0.0015

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.009	4	0.002	0.93	0.47	2.87
Within Groups	0.049	20	0.002			
Total	0.058	24				

A-1.14 Sibutramine at 5.0 ppm

	t_m (min)					Anova: Single Factor SUMMARY				
						Groups	*Count*	*Sum*	*Average*	*Variance*
Day 1	7.823	7.843	7.884	7.821	7.852	**Day 1**	5	39.22	7.845	0.00066
Day 2	7.837	7.841	7.838	7.874	7.843	**Day 2**	5	39.23	7.847	0.00024
Day 3	7.829	7.855	7.853	7.836	7.851	**Day 3**	5	39.22	7.845	0.00013
Day 4	7.834	7.866	7.841	7.889	7.851	**Day 4**	5	39.28	7.856	0.00048
Day 5	7.839	7.872	7.876	7.858	7.882	**Day 5**	5	39.33	7.865	0.00030

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.002	4	0.0004	1.15	0.36	2.87
Within Groups	0.007	20	0.0004			
Total	0.009	24				

A-1.15 Sibutramine at 30.0 ppm

	t_m (min)					Anova: Single Factor SUMMARY				
						Groups	*Count*	*Sum*	*Average*	*Variance*
Day 1	7.790	7.859	7.870	7.854	7.893	**Day 1**	5	39.27	7.853	0.0015
Day 2	7.785	7.869	7.832	7.883	7.834	**Day 2**	5	39.20	7.841	0.0015
Day 3	7.795	7.852	7.877	7.885	7.839	**Day 3**	5	39.25	7.850	0.0013
Day 4	7.855	7.803	7.835	7.876	7.823	**Day 4**	5	39.19	7.838	0.00080
Day 5	7.823	7.87	7.884	7.895	7.862	**Day 5**	5	39.33	7.867	0.00076

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.003	4	0.0006	0.56	0.70	2.87
Within Groups	0.023	20	0.0012			
Total	0.027	24				

Table A-2 Data of the measured concentration and ANOVA satistical analysis of five analytes at LOQ, 5.0 and 30.0 ppm

A-2.1 Phentermine at 0.45 ppm

the measured concentration						Anova: Single Factor SUMMARY				
						Groups	*Count*	*Sum*	*Average*	*Variance*
Day 1	0.41	0.45	0.44	0.43	0.42	**Day 1**	5	2.14	0.43	0.00022
Day 2	0.42	0.45	0.44	0.42	0.43	**Day 2**	5	2.15	0.43	0.00017
Day 3	0.44	0.45	0.44	0.45	0.45	**Day 3**	5	2.22	0.44	0.000058
Day 4	0.43	0.43	0.46	0.45	0.44	**Day 4**	5	2.20	0.44	0.00018
Day 5	0.42	0.46	0.46	0.45	0.44	**Day 5**	5	2.23	0.45	0.00020

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.001	4	0.0003	1.80	0.17	2.87
Within Groups	0.003	20	0.0002			
Total	0.005	24				

A-2.2 Phentermine at 5.0 ppm

the measured concentration						Anova: Single Factor SUMMARY				
						Groups	*Count*	*Sum*	*Average*	*Variance*
Day 1	5.01	4.93	4.85	4.72	5.17	**Day 1**	5	24.68	4.94	0.029
Day 2	5.25	5.09	5.06	5.32	5.18	**Day 2**	5	25.90	5.18	0.012
Day 3	5.14	4.86	5.25	4.84	5.24	**Day 3**	5	25.33	5.07	0.042
Day 4	4.82	5.16	5.10	4.77	5.09	**Day 4**	5	24.94	4.99	0.032
Day 5	5.14	4.86	5.10	5.33	5.10	**Day 5**	5	25.52	5.10	0.028

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.18	4	0.046	1.61	0.21	2.87
Within Groups	0.57	20	0.029			
Total	0.76	24				

A-2.3 Phentermine at 30.0 ppm

the measured concentration						Anova: Single Factor SUMMARY				
						Groups	*Count*	*Sum*	*Average*	*Variance*
Day 1	28.43	30.49	31.58	28.68	29.86	**Day 1**	5	149.02	29.80	1.7
Day 2	31.21	30.57	31.56	30.61	29.08	**Day 2**	5	153.03	30.61	0.90
Day 3	31.12	29.93	32.68	31.51	30.18	**Day 3**	5	155.43	31.09	1.2
Day 4	30.34	29.34	31.33	33.06	32.92	**Day 4**	5	156.99	31.40	2.6
Day 5	32.31	30.64	29.35	29.90	30.61	**Day 5**	5	152.81	30.56	1.2

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	7.3	4	1.83	1.20	0.34	2.87
Within Groups	31	20	1.53			
Total	38	24				

A-2.4 Pseudoephedrine at 0.50 ppm

the measured concentration						Anova: Single Factor SUMMARY				
						Groups	*Count*	*Sum*	*Average*	*Variance*
Day 1	0.47	0.51	0.48	0.49	0.53	**Day 1**	5	2.48	0.50	0.00048
Day 2	0.49	0.49	0.48	0.49	0.50	**Day 2**	5	2.45	0.49	0.000076
Day 3	0.50	0.48	0.49	0.49	0.49	**Day 3**	5	2.44	0.49	0.000076
Day 4	0.48	0.49	0.54	0.52	0.52	**Day 4**	5	2.55	0.51	0.00060
Day 5	0.50	0.52	0.51	0.51	0.52	**Day 5**	5	2.56	0.51	0.000076

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.002	4	0.0006	2.32	0.09	2.87
Within Groups	0.005	20	0.0003			
Total	0.008	24				

A-2.5 Pseudoephedrine at 5.0 ppm

the measured concentration						Anova: Single Factor SUMMARY				
						Groups	*Count*	*Sum*	*Average*	*Variance*
Day 1	4.94	4.79	5.16	4.92	4.93	**Day 1**	5	24.74	4.95	0.018
Day 2	5.08	5.22	4.91	4.99	5.21	**Day 2**	5	25.42	5.08	0.019
Day 3	4.76	4.97	5.07	4.72	5.02	**Day 3**	5	24.53	4.91	0.025
Day 4	5.05	4.76	4.73	4.80	5.13	**Day 4**	5	24.46	4.89	0.033
Day 5	4.83	5.04	5.08	4.75	4.95	**Day 5**	5	24.64	4.93	0.019

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.12	4	0.030	1.29	0.31	2.87
Within Groups	0.46	20	0.023			
Total	0.57	24				

A-2.6 Pseudoephedrine at 30.0 ppm

the measured concentration						Anova: Single Factor SUMMARY				
						Groups	*Count*	*Sum*	*Average*	*Variance*
Day 1	29.19	31.06	30.93	32.10	31.09	**Day 1**	5	154.36	30.87	1.1
Day 2	28.69	30.68	28.97	31.43	29.29	**Day 2**	5	149.06	29.81	1.4
Day 3	29.68	29.23	29.19	31.38	30.55	**Day 3**	5	150.03	30.01	0.89
Day 4	28.08	29.00	28.23	30.43	31.45	**Day 4**	5	147.19	29.44	2.1
Day 5	30.72	29.41	31.07	29.70	31.68	**Day 5**	5	152.57	30.51	0.90

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	6.5	4	1.62	1.26	0.32	2.87
Within Groups	26	20	1.29			
Total	32	24				

A-2.7 Ephedrine at 0.50 ppm

the measured concentration					
Day 1	0.47	0.46	0.47	0.50	0.47
Day 2	0.51	0.50	0.47	0.46	0.51
Day 3	0.47	0.49	0.48	0.51	0.48
Day 4	0.51	0.49	0.51	0.50	0.51
Day 5	0.48	0.47	0.51	0.49	0.51

Anova: Single Factor
SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Day 1	5	2.38	0.48	0.00016
Day 2	5	2.45	0.49	0.00039
Day 3	5	2.43	0.49	0.00016
Day 4	5	2.51	0.50	0.000057
Day 5	5	2.46	0.49	0.00023

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.002	4	0.0004	2.08	0.12	2.87
Within Groups	0.004	20	0.0002			
Total	0.006	24				

A-2.8 Ephedrine at 5.0 ppm

the measured concentration					
Day 1	5.16	5.02	5.14	4.97	4.99
Day 2	4.90	5.03	5.07	4.93	5.00
Day 3	5.13	5.09	5.15	5.22	5.18
Day 4	5.03	5.08	4.68	4.91	4.88
Day 5	5.07	4.72	4.92	5.19	5.08

Anova: Single Factor
SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Day 1	5	25.27	5.05	0.0078
Day 2	5	24.92	4.98	0.0047
Day 3	5	25.77	5.15	0.0023
Day 4	5	24.57	4.91	0.025
Day 5	5	24.96	4.99	0.032

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.2	4	0.04	2.79	0.05	2.87
Within Groups	0.3	20	0.01			
Total	0.5	24				

A-2.9 Ephedrine at 30.0 ppm

the measured concentration					
Day 1	32.07	29.37	31.65	28.61	31.13
Day 2	31.90	29.51	30.35	31.50	31.33
Day 3	30.14	29.70	29.40	29.40	31.68
Day 4	30.01	29.72	30.83	31.95	29.48
Day 5	30.28	29.70	32.55	31.55	32.72

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Day 1	5	152.84	30.57	2.3
Day 2	5	154.59	30.92	0.94
Day 3	5	150.33	30.07	0.91
Day 4	5	151.98	30.40	1.0
Day 5	5	156.80	31.36	1.8

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	4.9	4	1.2	0.89	0.49	2.9
Within Groups	27.7	20	1.4			
Total	33	24				

A-2.10 Fenfluramine at 0.55 ppm

the measured concentration					
Day 1	0.55	0.55	0.58	0.57	0.56
Day 2	0.55	0.53	0.55	0.58	0.57
Day 3	0.54	0.55	0.57	0.57	0.57
Day 4	0.56	0.55	0.56	0.54	0.57
Day 5	0.57	0.56	0.57	0.59	0.57

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Day 1	5	2.82	0.56	0.00014
Day 2	5	2.79	0.56	0.00031
Day 3	5	2.81	0.56	0.00016
Day 4	5	2.79	0.56	0.00011
Day 5	5	2.86	0.57	0.00010

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.00071	4	0.00018	1.08	0.39	2.87
Within Groups	0.0033	20	0.00016			
Total	0.0040	24				

A-2.11 Fenfluramine at 5.0 ppm

the measured concentration					
Day 1	4.82	4.56	5.02	4.96	4.89
Day 2	4.91	5.10	4.94	5.10	5.03
Day 3	5.23	5.03	5.19	4.90	4.84
Day 4	4.83	5.18	4.88	4.74	4.81
Day 5	5.07	4.97	4.98	5.17	5.09

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Day 1	5	24.25	4.85	0.032
Day 2	5	25.10	5.02	0.0080
Day 3	5	25.20	5.04	0.030
Day 4	5	24.43	4.89	0.029
Day 5	5	25.27	5.05	0.0068

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.18	4	0.04	2.12	0.12	2.87
Within Groups	0.42	20	0.02			
Total	0.60	24				

A-2.12 Fenfluramine at 30.0 ppm

the measured concentration					
Day 1	30.81	30.62	29.85	30.10	30.81
Day 2	30.53	30.17	31.04	31.37	31.57
Day 3	30.00	29.64	31.40	30.21	29.70
Day 4	30.67	29.60	30.59	31.75	31.28
Day 5	30.48	31.41	30.73	31.50	31.35

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Day 1	5	152.18	30.44	0.19
Day 2	5	154.69	30.94	0.34
Day 3	5	150.94	30.19	0.51
Day 4	5	153.89	30.78	0.66
Day 5	5	155.47	31.09	0.21

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.7	4	0.68	1.80	0.17	2.87
Within Groups	7.6	20	0.38			
Total	10	24				

A-2.13 Sibutramine at 1.0 ppm

the measured concentration						Anova: Single Factor SUMMARY				
						Groups	*Count*	*Sum*	*Average*	*Variance*
Day 1	0.93	0.96	0.96	0.92	0.90	**Day 1**	5	4.68	0.94	0.00063
Day 2	0.98	0.93	0.95	0.98	0.93	**Day 2**	5	4.77	0.95	0.00048
Day 3	0.95	0.97	0.98	0.96	0.99	**Day 3**	5	4.85	0.97	0.00020
Day 4	0.95	0.98	0.96	0.98	0.94	**Day 4**	5	4.81	0.96	0.00041
Day 5	0.97	0.96	0.96	0.99	0.97	**Day 5**	5	4.85	0.97	0.00014

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.0039	4	0.0010	2.66	0.06	2.87
Within Groups	0.0074	20	0.0004			
Total	0.011	24				

A-2.14 Sibutramine at 5.0 ppm

the measured concentration						Anova: Single Factor SUMMARY				
						Groups	*Count*	*Sum*	*Average*	*Variance*
Day 1	4.63	4.79	4.97	4.65	4.96	**Day 1**	5	24.01	4.80	0.027
Day 2	4.85	4.89	4.93	4.92	5.13	**Day 2**	5	24.73	4.95	0.012
Day 3	5.09	5.00	5.33	5.05	5.19	**Day 3**	5	25.66	5.13	0.018
Day 4	4.88	5.30	4.92	4.68	4.76	**Day 4**	5	24.54	4.91	0.057
Day 5	5.27	4.84	5.19	5.02	4.87	**Day 5**	5	25.19	5.04	0.036

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.32	4	0.079	2.64	0.06	2.87
Within Groups	0.60	20	0.030			
Total	0.92	24				

A-2.15 Sibutramine at 30.0 ppm

	the measured concentration					Anova: Single Factor SUMMARY				
						Groups	*Count*	*Sum*	*Average*	*Variance*
Day 1	29.26	29.95	30.62	28.49	28.59	**Day 1**	5	146.90	29.38	0.83
Day 2	29.63	29.49	30.50	31.20	30.68	**Day 2**	5	151.50	30.30	0.53
Day 3	28.56	29.26	30.63	28.24	28.14	**Day 3**	5	144.84	28.97	1.1
Day 4	29.12	27.61	29.35	30.50	30.08	**Day 4**	5	146.66	29.33	1.2
Day 5	29.72	28.38	29.96	28.22	31.19	**Day 5**	5	147.47	29.49	1.5

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	4.8	4	1.2	1.17	0.35	2.87
Within Groups	21	20	1.0			
Total	25	24				

APPENDIX B

Figure B-1 UV-Visible spectra of five anti-obesity drugs with the maximum absorbance at 214 nm

Figure B-1.1 Phentermine

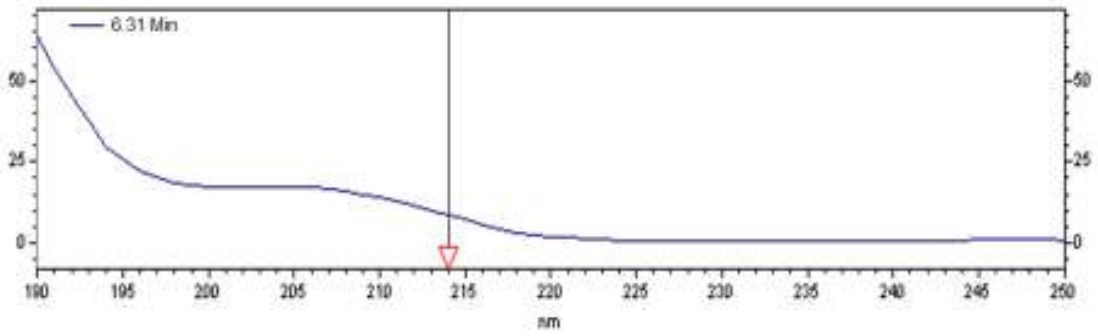


Figure B-1.2 Sibutramine

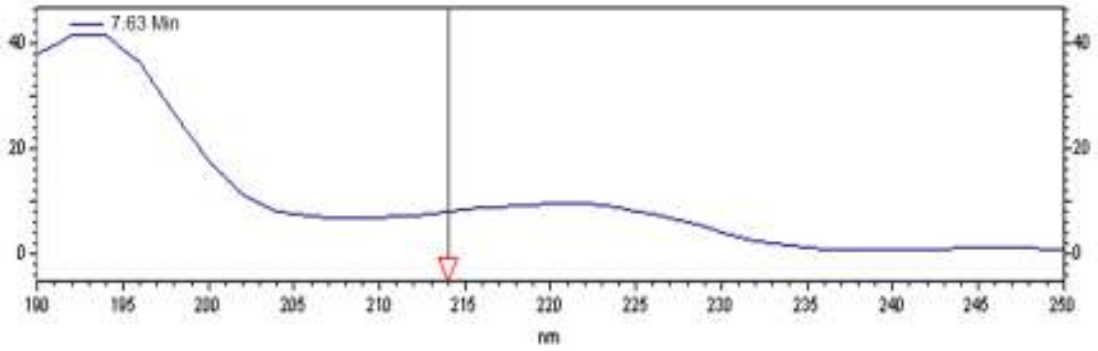


Figure B-1.3 Ephedrine

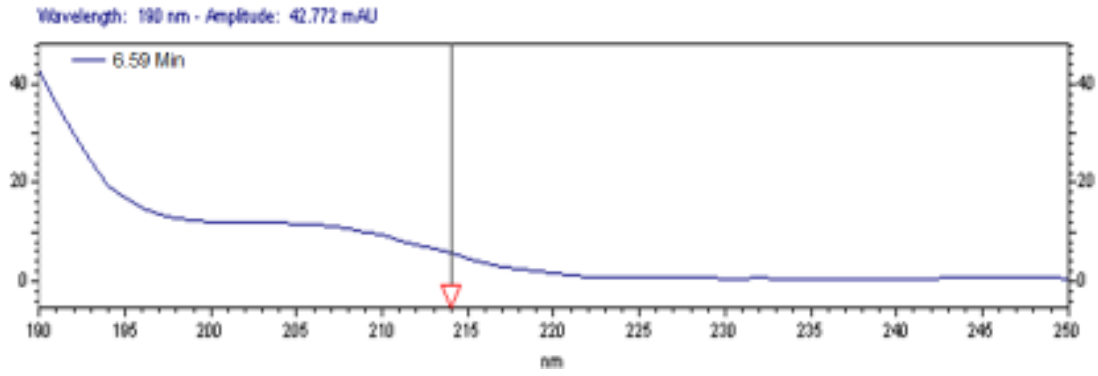


Figure B-1.4 Pseudoephedrine

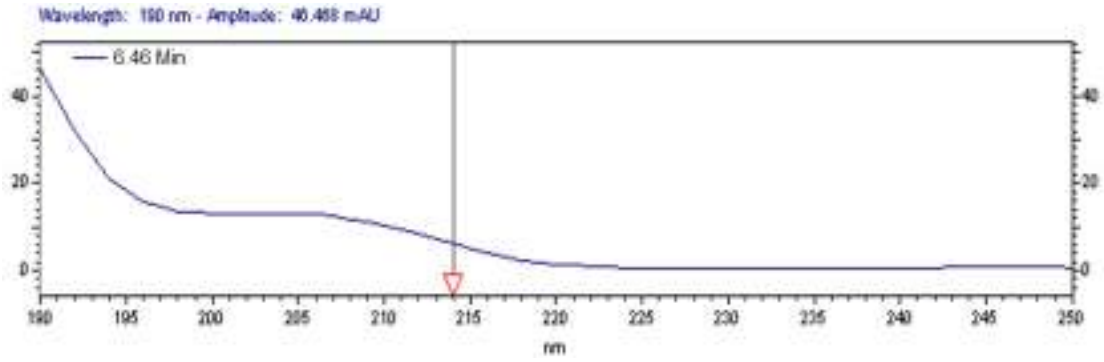


Figure B-1.5 Fenfluramine

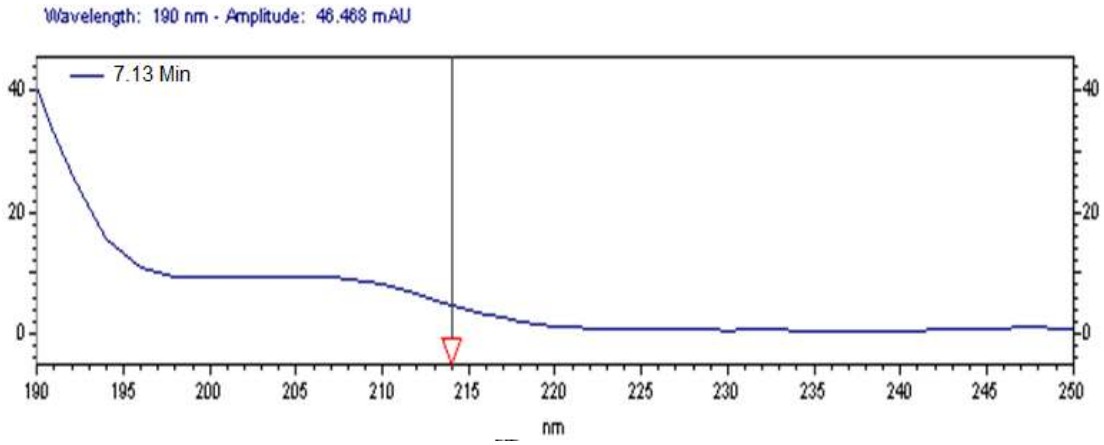


Figure B-2 CZE electropherograms for analysis of anti-obesity drugs adulterated in dietary suppremen

Figure B-2.1a In comparison in order to t_m for standard solution with real sample C1

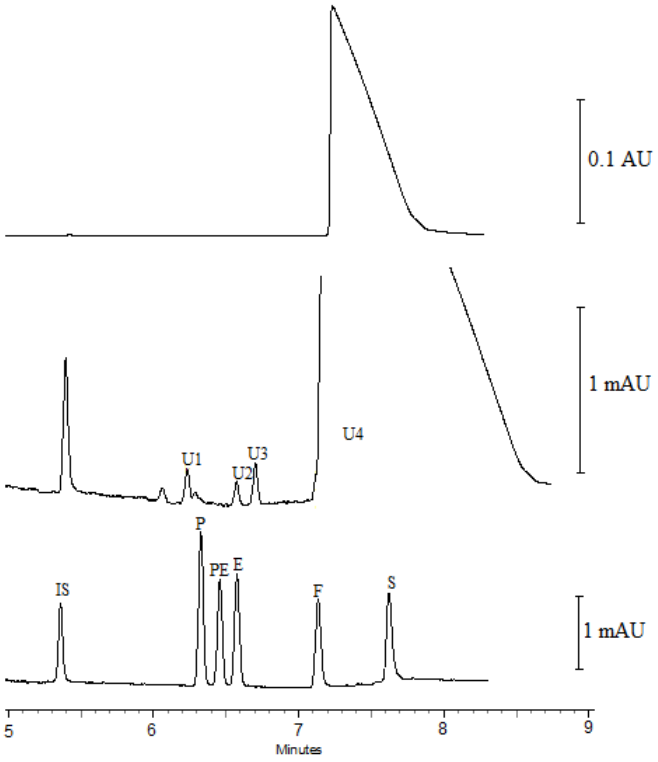
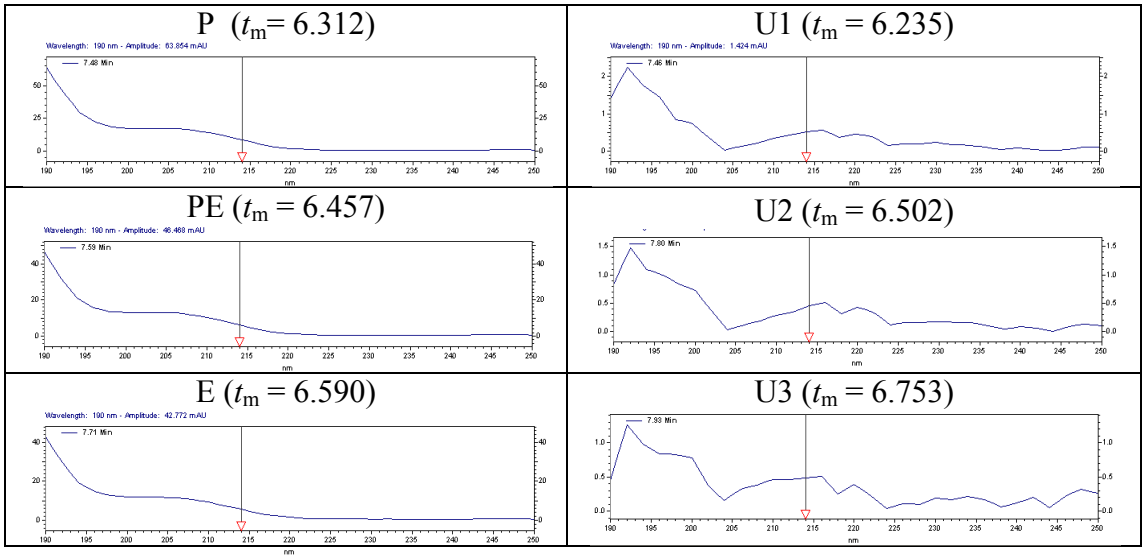


Figure B-2.1b In comparison in order to UV spectra for standard solution with real sample C1



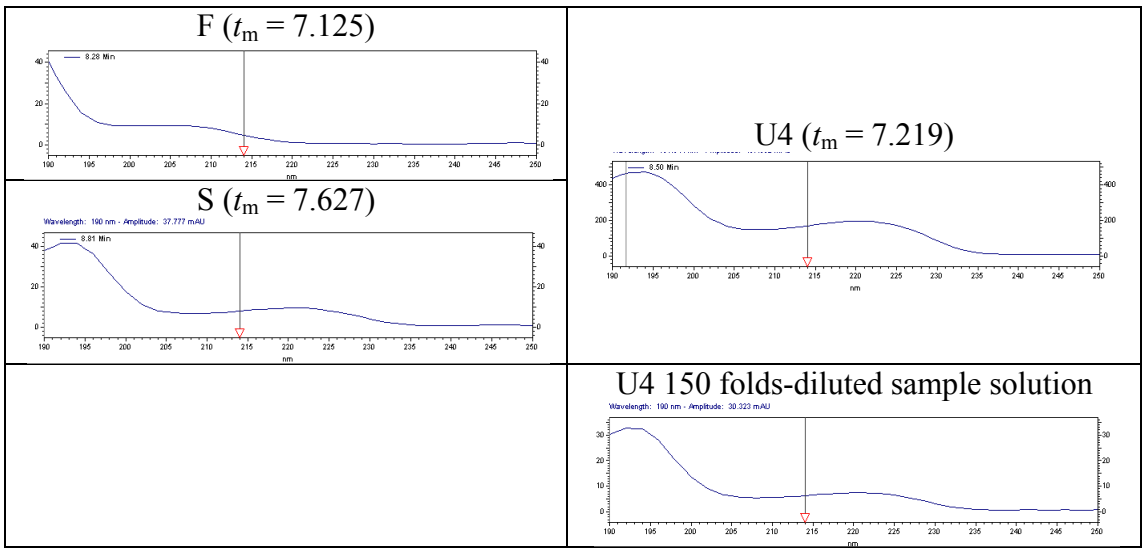


Figure B-2.1c CZE electropherograms for analysis of C1. a1) standard solution, a2) 150 folds-diluted sample solution, b3) 150 folds-diluted sample solution spiked with S.

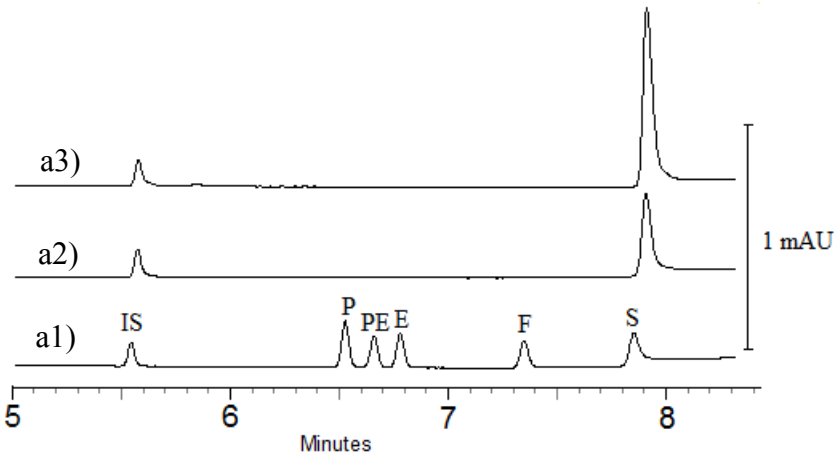


Figure B-2.2a In comparison in order to t_m for standard solution with real sample C2

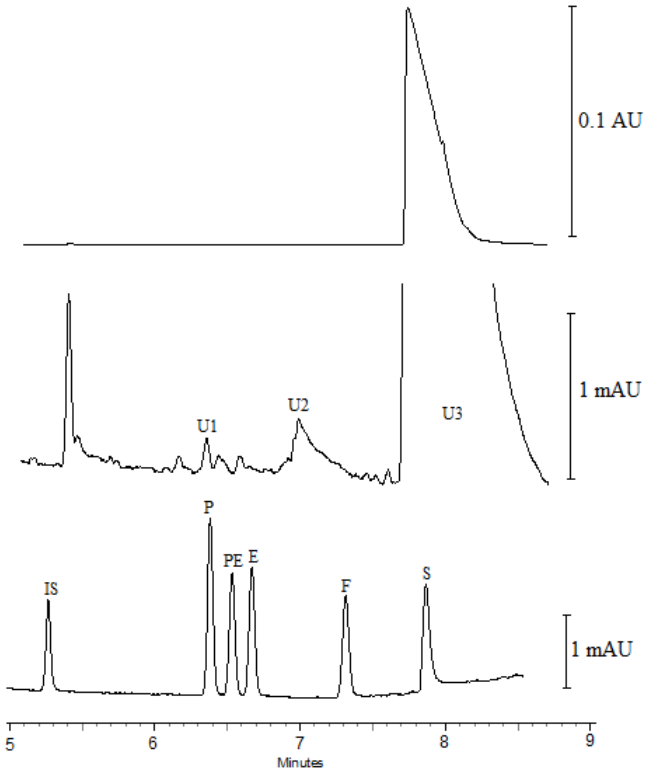
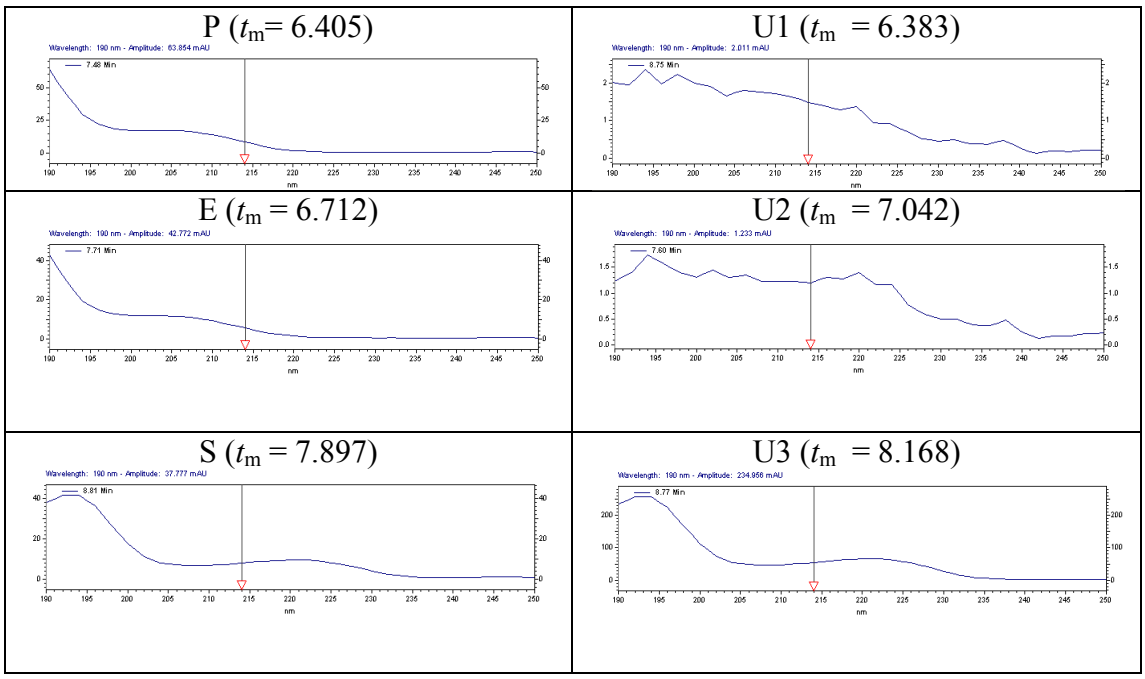


Figure B-2.2b In comparison in order to UV spectra for standard solution with real sample C2



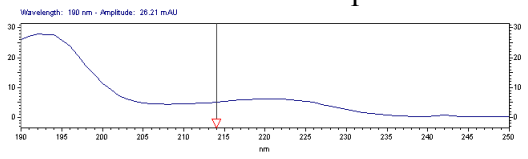
	U3 150 folds-diluted sample solution 

Figure B-2.2c CZE electropherograms for analysis of C1. a1) standard solution, a2) 150 folds-diluted sample solution, b3) 150 folds-diluted sample solution spiked with S.

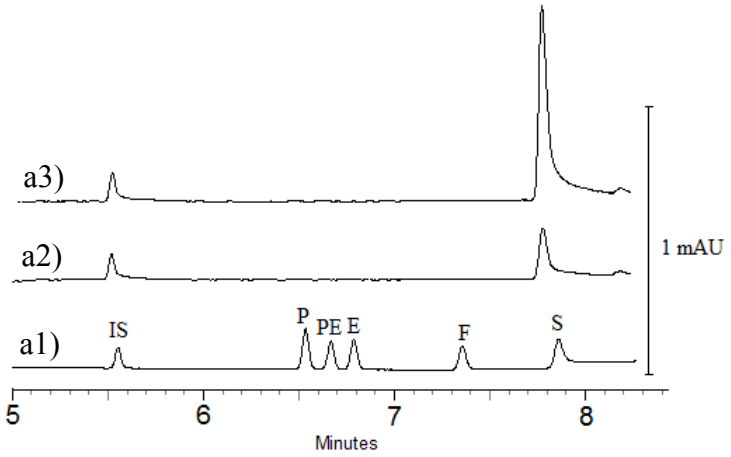


Figure B-2.3a In comparison in order to t_m for standard solution with real sample C3

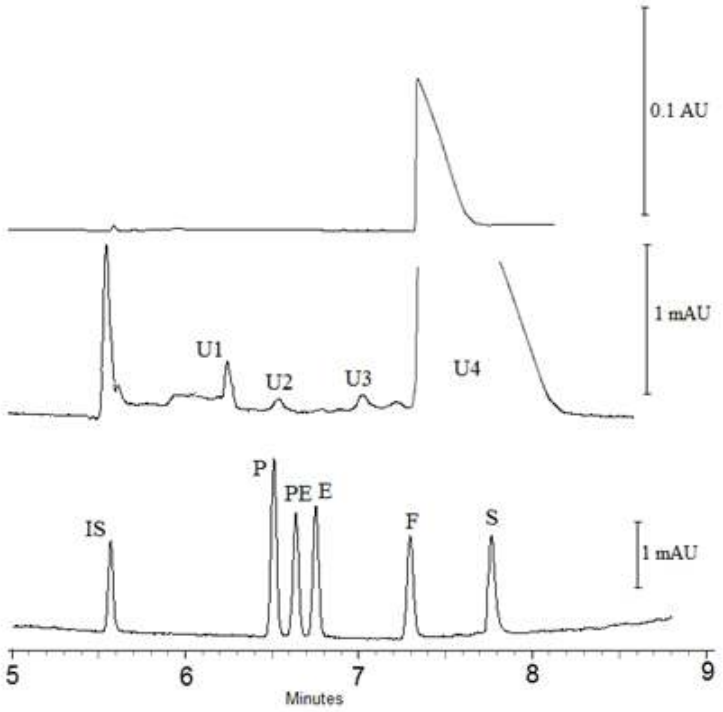


Figure B-2.3b In comparison in order to UV spectra for standard solution with real sample C3

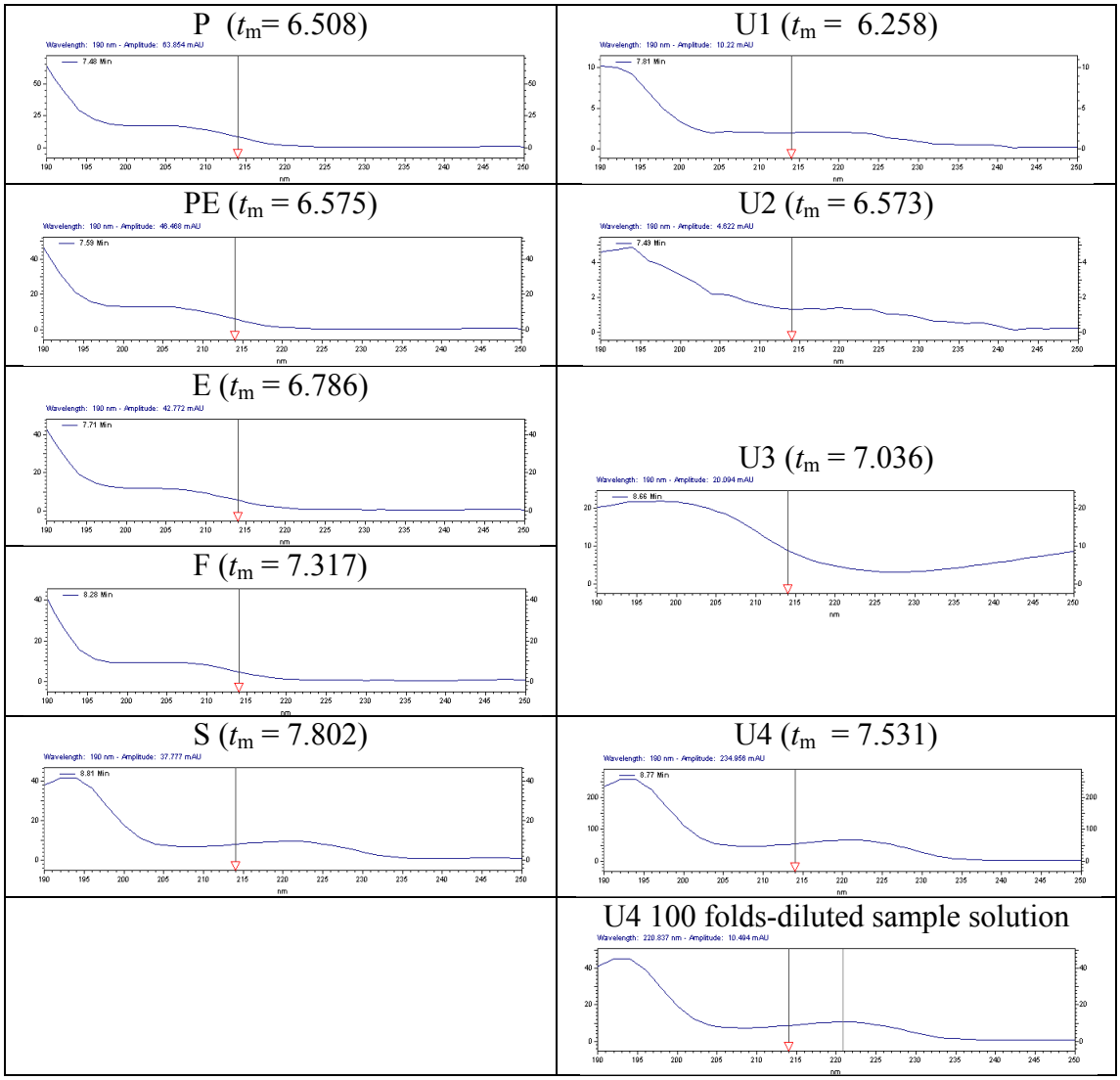


Figure B-2.3c CZE electropherograms for analysis of C3. a1) standard solution, a2) 100 folds-diluted sample solution, b3) 100 folds-diluted sample solution spiked with S.

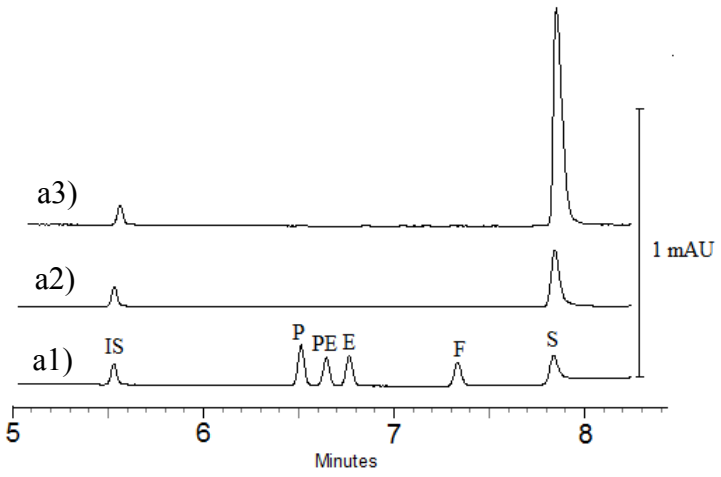


Figure B-2.4a In comparison in order to t_m for standard solution with real sample C4

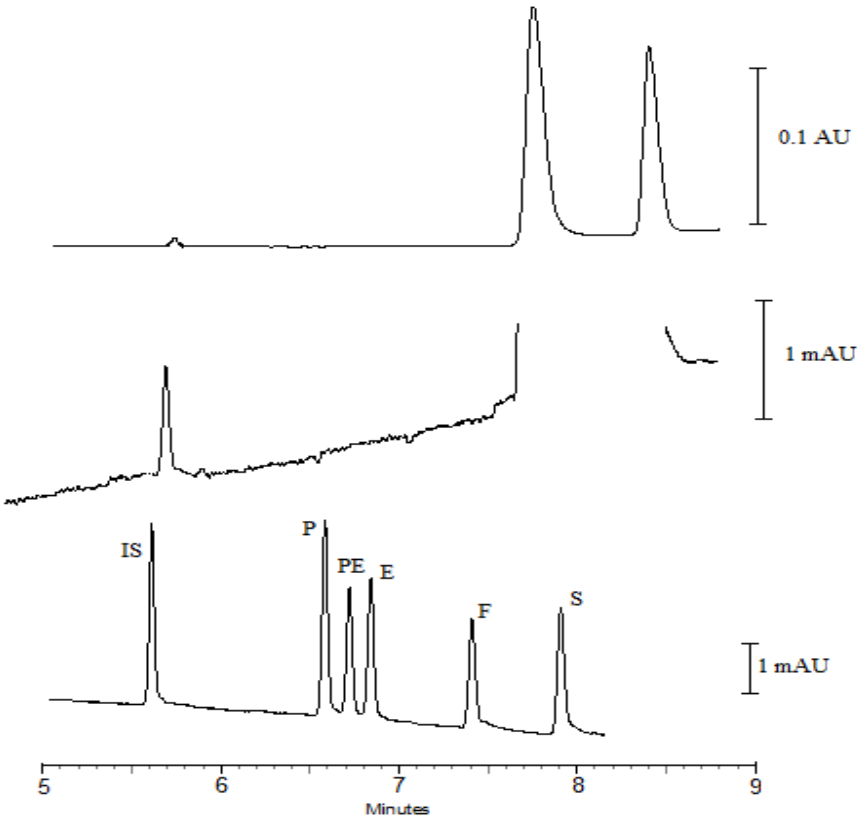


Figure B-2.4b In comparison in order to UV spectra for standard solution with real sample C4

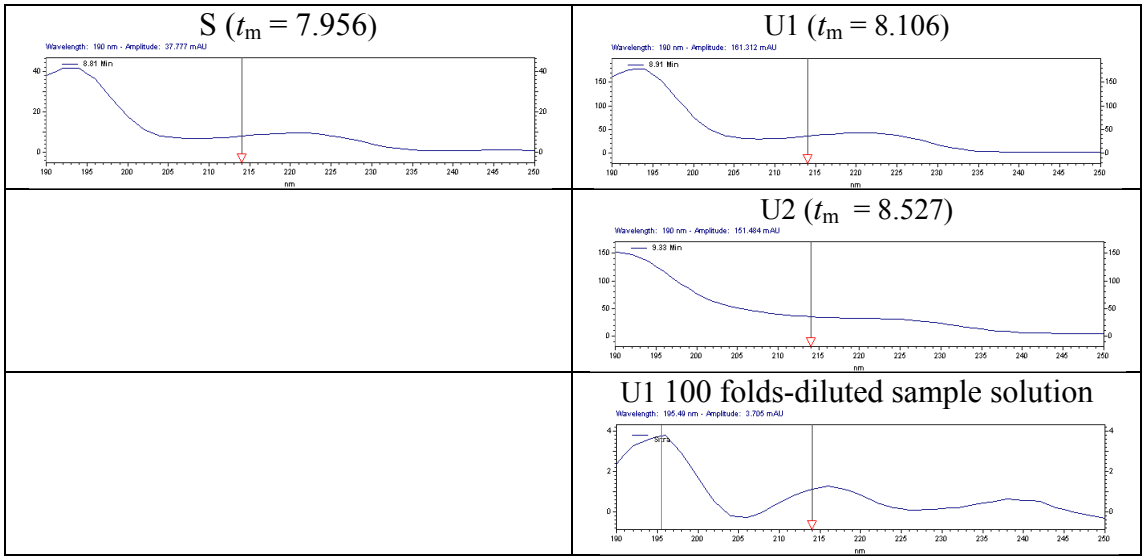


Figure B-2.4c CZE electropherograms for analysis of C4. a1) standard solution, a2) 100 folds-diluted sample solution, b3) 100 folds-diluted sample solution spiked with S.

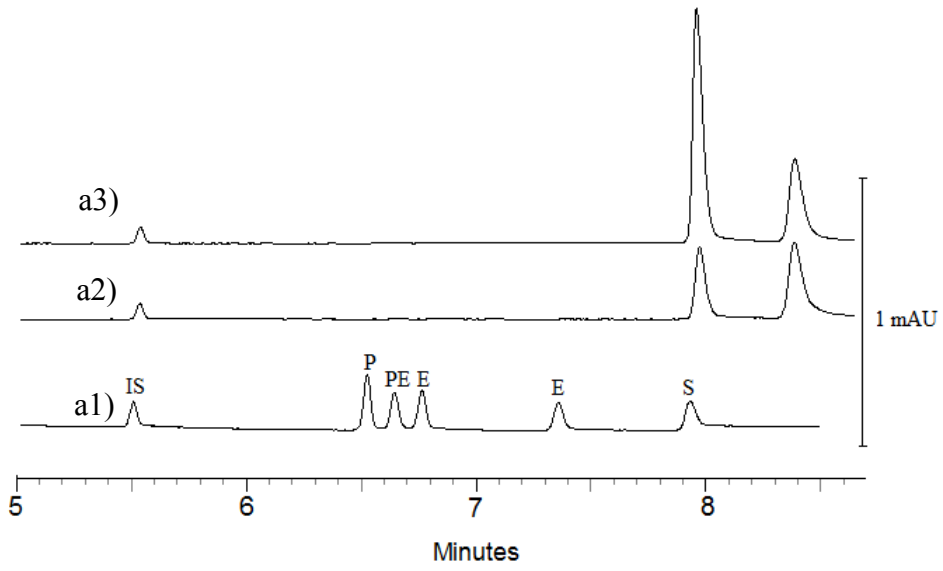


Figure B-3 CZE electropherograms for analysis of anti-obesity drugs adulterated in dietary suppreme nt (not detect)

Figure B-3.1a In comparison in order to t_m for standard solution with real sample C5

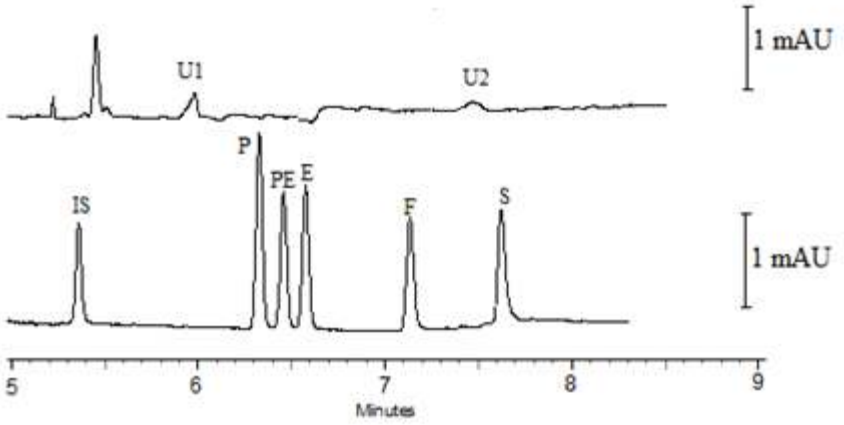


Figure B-3.1b In comparison in order to UV spectra for standard solution with real sample C5

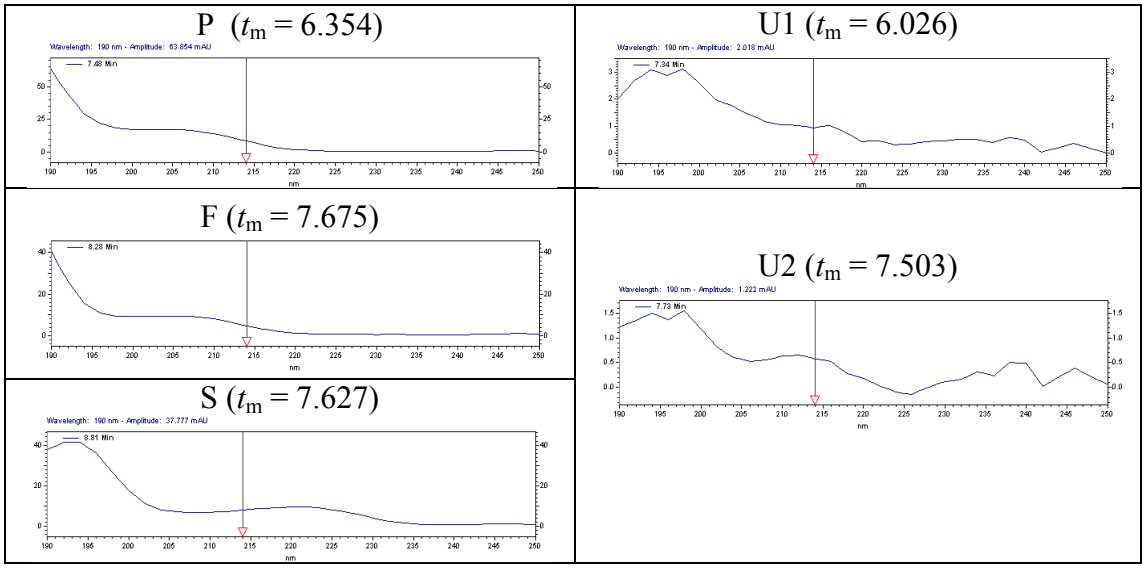


Figure B-3.2a In comparison in order to t_m for standard solution with real sample C6

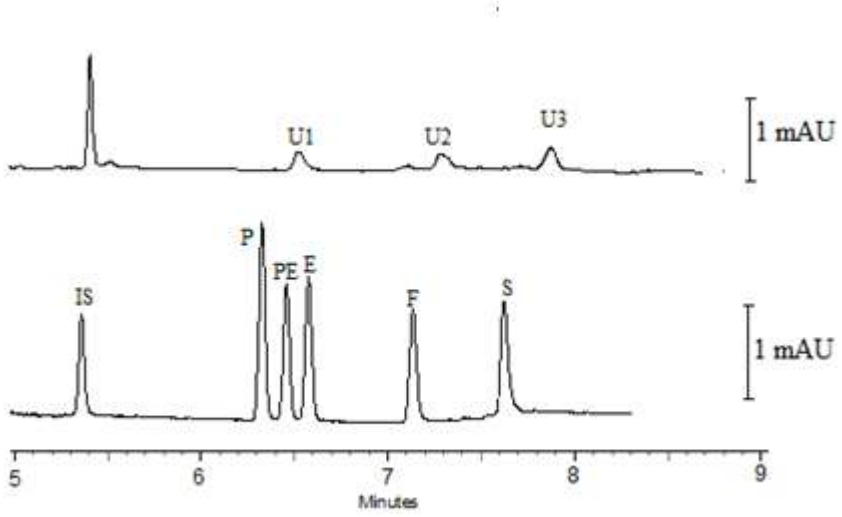


Figure B-3.2b In comparison in order to UV spectra for standard solution with real sample C6

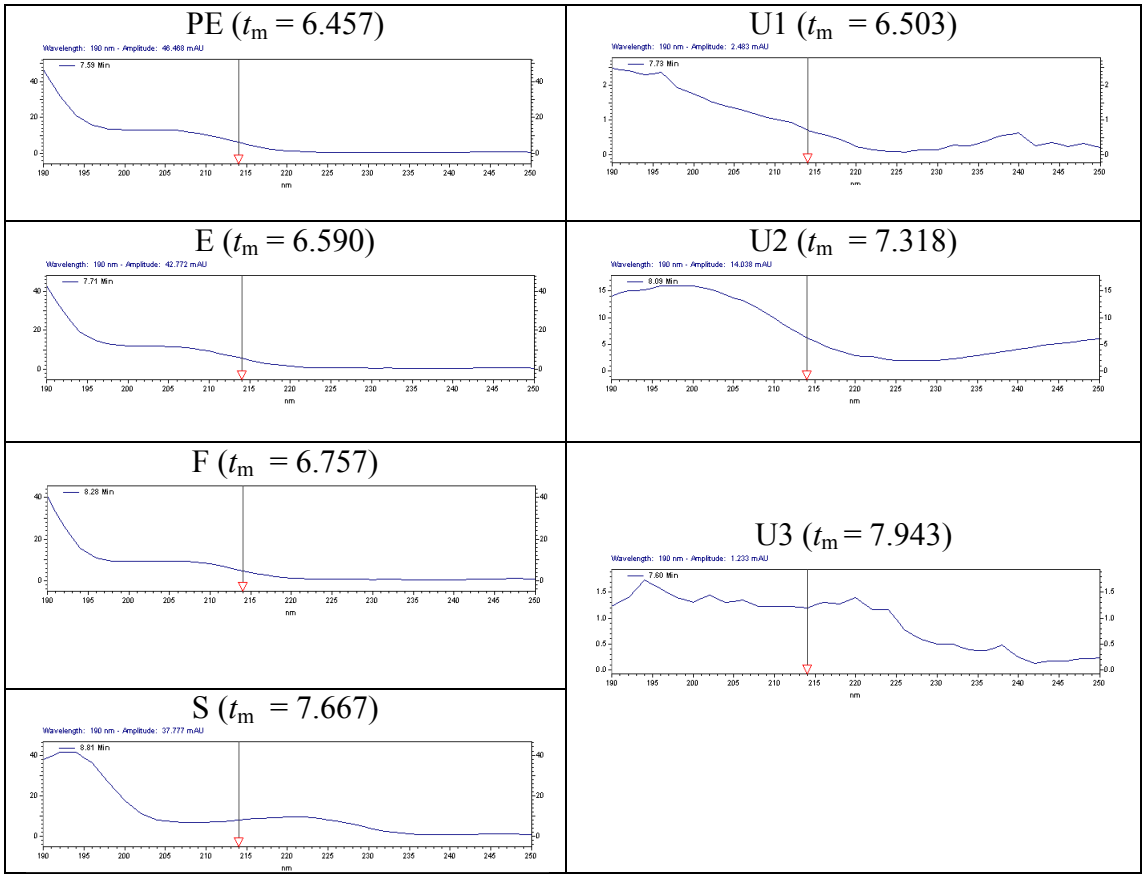


Figure B-3.3a In comparison in order to t_m for standard solution with real sample C7

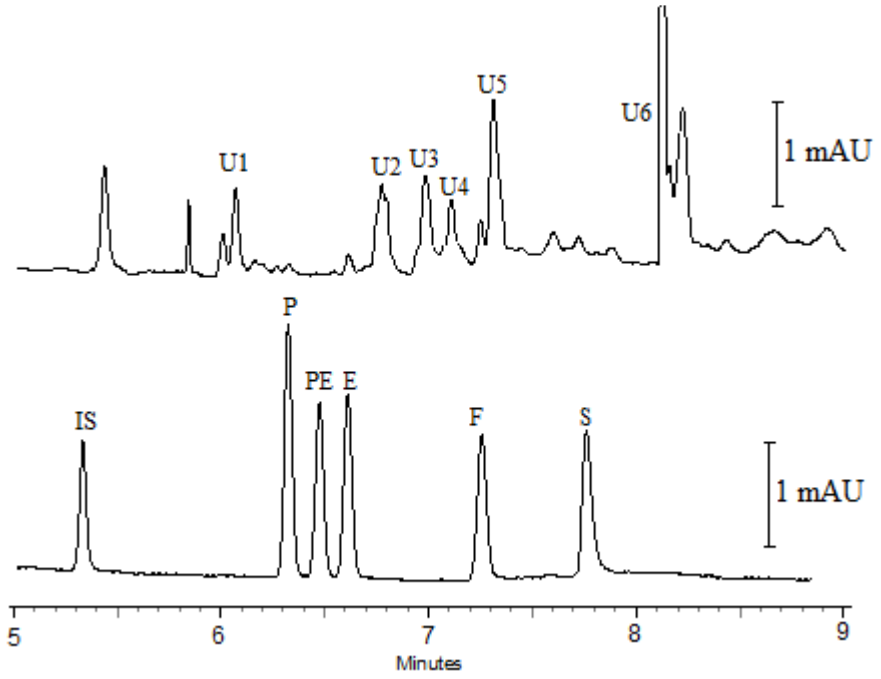
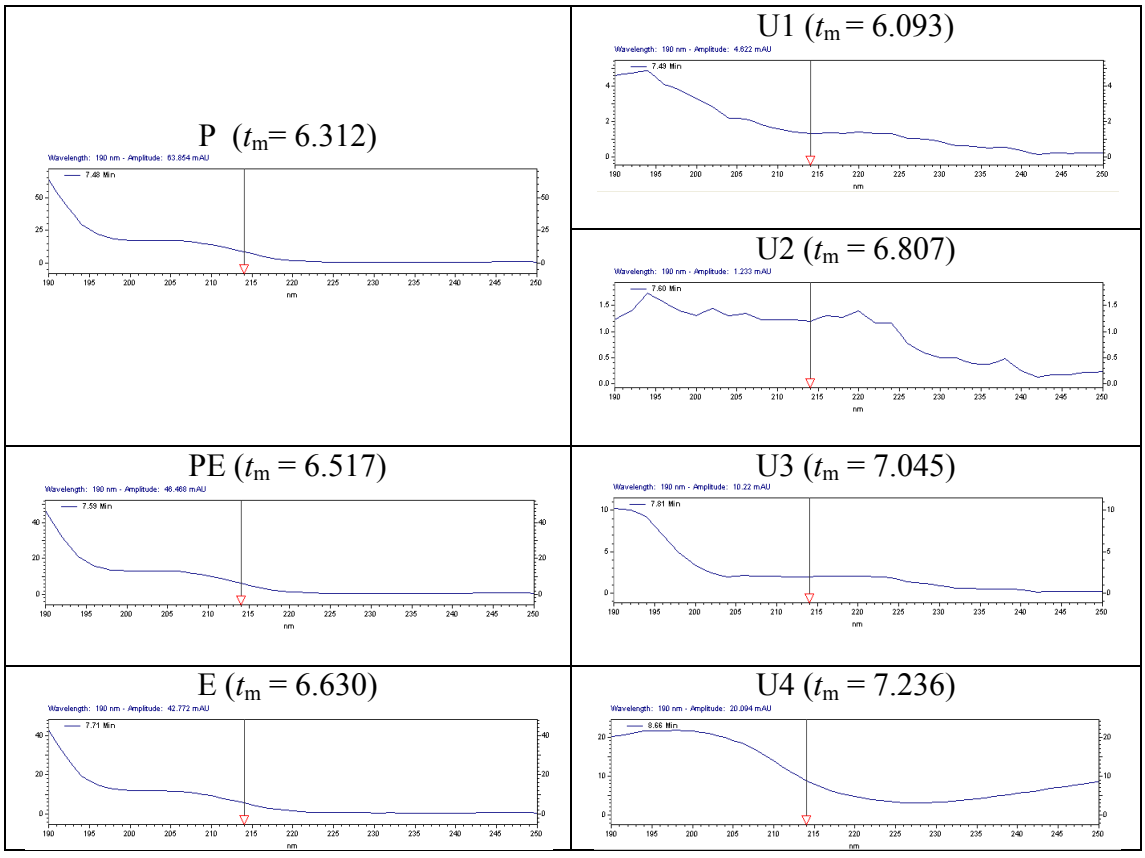


Figure B-3.3b In comparison in order to UV spectra for standard solution with real sample C7



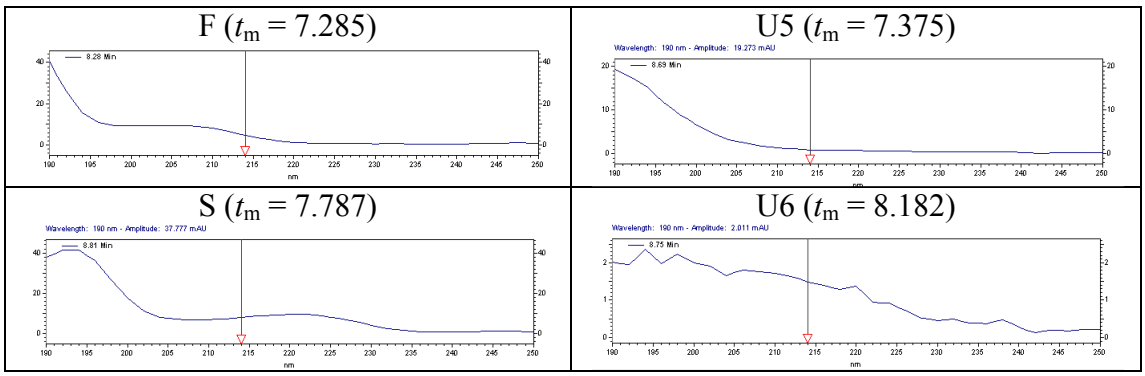
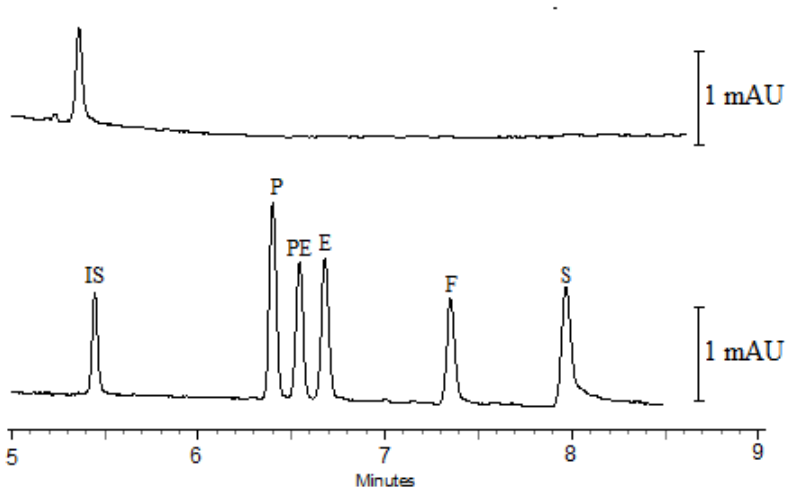


Figure B-3.4a In comparison in order to t_m for standard solution with real sample C8



VITA

Mr. Natthaphong Chomvana was born on July 1st, 1987, in Phra Nakhon Sri Ayutthaya, Thailand. He graduated with a Bachelor's degree of Science in Chemistry from King Mongkut's Institute of Technology Ladkrabang in 2010. Afterwards, he continued his studies in a Master program in Chemistry, Faculty of Science, Chulalongkorn University, and graduated with a Master's degree of Science in 2013.