DOKUZ EYLÜL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

OPTIMIZATION OF LC-MS/MS INSTRUMENTAL ANALYSIS CONDITIONS FOR MEASUREMENT OF ESTROGENIC HORMONES

by
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İZMİR

OPTIMIZATION OF LC-MS/MS INSTRUMENTAL ANALYSIS CONDITIONS FOR MEASUREMENT OF ESTROGENIC HORMONES

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M.Sc THESIS EXAMINATION RESULT FORM

We have read the thesis entitled **"OPTIMIZATION OF LC-MS/MS INSTRUMENTAL ANALYSIS CONDITIONS FOR MEASUREMENT OF ESTROGENIC HORMONES"** completed by **BİNNAZ ŞAHİNTÜRK** under supervision of **PROF. DR. İLGİ K. KAPDAN** and we certify that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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OPTIMIZATION OF LC-MS/MS INSTRUMENTAL ANALYSIS CONDITIONS FOR MEASUREMENT OF ESTROGENIC HORMONES

ABSTRACT

Endocrine Disrupting Chemicals are defined as substances that elicit the same chemical reactions as natural hormones, prevent naturally occurring hormones from affecting cells in the usual way by blocking the cell receptors, elicit unusual or abnormal reactions in cells. Estrogenic hormones are one of EDC present in natural waters and wastewaters. Their effect on human and animals is not well known yet. But, it could be a main concern to protect human and animal life soon when their effects on growth and reproductive system are clearly understood. It is evident that their concentration in the water is very low. Therefore, it is not easy to detect their existence in water. There is an urgent need to develop methods to be able to measure very low concentrations of these chemicals at ng/L or pg/L levels.

By considering this fact, the aim of this thesis was to develop an instrumental method for the measurement of estrogenic hormones as E1, E2, E3 and EE2 in LC-MS/MS. For this purpose, the study was designed as two stage optimization of elution conditions and instrumental operational conditions. The first stage was i) pre optimization of mobile phase, injection solution compositions and flow for peak symmetry and resolution factor by using central composite design, ii) improvement of integration area with the addition of alkaline solution (Ammonia) into mobile phase. Ionization was substantially improved by the addition of alkaline solution. Therefore, second stage optimization was conducted for the new mobile phase composition with ammonia solution. The latter stage designed as i) determination of ranges of operation parameters of LC-MS/MS ii) improvement of optimization conditions for LC Elution at determined ranges of operation parameters of LC-MS/MS by using Box Behnken response surface method, iii) improvement of optimized conditions for LC-MS/MS operation for the determined new elution parameters. Peak symmetry, resolution factor and integration area of hormones in chromatograms were substantially improved by the final optimization. Linear range

of calibration curve developed at the optimal conditions were between 5 ng/L-30 ng/L which means even 5 ng/L estrogenic hormone concentration can be measured by this method.

Keywords: Estrogenic hormones, endocrine disrupting chemicals, response surface method, LC-MS/MS.

ÖSTROJENİK HORMONLARIN ÖLÇÜMÜNDE LC-MS/MS CİHAZ ANALİZ ŞARTLARININ OPTİMİZASYONU

ÖZ

Endokrin bozucu kimyasallar, normal hormonların kimyasal reaksiyonlarını taklit ederek, ilgili reseptörlere bağlanarak hormon taklidi yapan ya da aşırı hormon salınımına sebep olarak anormalliklerin meydana gelmesine sebep olan kimyasallar olarak tanımlanmaktadır. Estrojenik hormonlar bu grupta yer almakta olup, yüzeysel ve atıksularda bulunmaktadırlar. İnsan sağlığı üzerine etkileri henüz tam olarak bilinmemektedir. Ancak, büyüme ve üreme sistemi üzerine etkileri anlaşıldığından insan ve hayvanları korumak için yakın zamanda önlemlerin alınması gereken kirleticiler olarak ortaya çıkacaktır. Estrojenik hormonların sudaki derişimlerinin çok düşük olmasından dolayı tespit edilmeleri kolay olmamaktadır. Bu nedenle, ng/L ve pg/L seviyelerinde ölçümlerin yapılabileciği metodların geliştirilmesi gereklidir.

Bu gerçekler göz önüne alınarak, bu tezin amacı östrojenik hormonlardan olan E1, E2, E3 ve EE2'nin LC-MS/MS cihazında ölçümlerine ilişkin enstrumental yöntemlerin geliştirilmesidir. Bu amaçla çalışmalar LC elüsyonun optimizasyonu ve enstrumental operasyon koşullarının optimizasyonu olmak üzere iki aşamada tasarlanmıştır. İlk aşama: i) pik simetrileri ve pik ayrım faktörleri için mobil fazın, enjeksiyon çözeltisi bileşiminin ve akışın merkezi kompozit dizayn yöntemiyle ön optimizasyonu gerçekleştirilmesi, ii) bazik amonyak çözeltisinin mobil faza eklenmesi ile alan değerlerinin iyileştirilmesidir. İyonizasyon bazik çözeltinin eklenmesi ile önemli derecede artmıştır. Bu nedenle, ikinci aşamada amonyak çözeltisi içeren mobil faz kompozisyonu optimize edilmiştir. Sonraki aşamada: i) LC-MS/MS'in çalışma parametrelerinin aralıklarının belirlenmesi, ii) belirlenen bu çalışma aralıklarında Box-Behnken deneysel tasarım yöntemi ile LC elüsyonunun iyileştirilmesi, iii) belirlenen yeni elüsyon parametreleri ile LC-MS/MS'in optimize edilen çalışma şartlarının geliştirilmesidir. Pik simetrisi, pik ayrım faktörü ve kromotogramlardaki hormonlara ilişkin alan değerlerinde son optimizasyon ile oldukça gelişme gözlenmiştir. Optimum koşullarda kalibrasyon eğrisinin lineer

aralığı 5 ng/L-30 ng/L dir ki bu yöntemle 5 ng/L hormon derişiminin ölçülebileceği anlamına gelmektedir.

Anahtar Kelimeler: Endokrin bozucu kimyasallar, yanıt yüzey yöntemi, östrojenik hormonlar, LC-MS/MS.

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CHAPTER ONE
INTRODUCTION

1.1 The Endocrine System and Hormones

The endocrine system is one of communication systems of the body and it has several glands which excrete hormones with different functions in animals regulating and integrating the function of different cells, body growth, reproduction, maintenance, homeostasis and metabolism are managed by the endocrine systems in both plants and animals. (Gill, Súilleabháin, Regan & Moran, 2007). The hypothalamus, pancreas, pineal body, thyroid, parathyroid, adrenals, and the reproductive organs (ovaries and testes), pituitary are the major glands of body (Singhal, Song, Johnson & Swift, 2010).

The role of hormones excreted by body glands (ductless), which called chemical messengers of the body, is to transfer information from one set of cells to another in order to coordinate and regulate functions and metabolism of the body. They are excreted into the bloodstream from glands and transported to receptors where they trigger responses (Singhal et al., 2010).

When the hormones reach target cells (or organs), active area of hormone and active area of target cells (bonding site) bind with each other. This process is called "a lock and key receptor binding" procedure. Hormones attach to the receptor and the effector site is altered which produces the desired response. The receptor sites have a very high affinity for a specific hormone meaning that only very low concentrations are required to get the response (Gill et al., 2007).

There are three classes of hormones that include peptides, amines and steroids (Figure 1.1). Peptides are the most extensive group of them. Amines are excreted from the adrenal medulla and the thyroid gland. Amines are derivative of tyrosine. Peptides molecules are excreted by the parathyroid, heart, stomach, pituitary, kidneys and liver. Steroids are excreted by the adrenal cortex, placenta and gonads, are lipids

derived from cholesterol. Differentiation, sexual determination and development are controlled by the steroid hormones and can be selected as androgens and estrogens. The major estrogens include estriol, 17-estradiol, estrone (female sex hormones), while the major androgens are testosterone (male sex hormone) and 5-dihydrotestosterone. 11-ketotestosterone is the main androgen in fish. The most importance of the androgens and estrogens are having central role in reproductivity (Singhal et al., 2010).

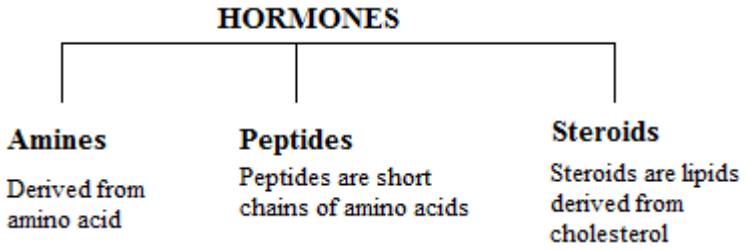


Figure 1.1 Classification of hormones.

1.2 Steroid Hormones

Steroid hormones are synthesized from cholesterol. Steroid hormones biosynthesis requires series of enzymes located in both endoplasmic reticulum and mitochondria. The free cholesterol transportation from the cytoplasm into mitochondria is the rate-limiting step of the process. The first reaction step which is occurred in mitochondria is transformation of cholesterol to pregnenolone by an enzyme in the inner membrane called CYP11A1. All steroid, hormones are synthesized from pregnenolone which is precursor however itself is not a hormone (Figure 1.2). Table 1.1 describes the enzymes required to synthesize the major classes of steroid hormones (Bowen, 2001).

Table 1.1 The enzymes required to synthesize the major classes of steroid hormones (Bowen, 2001).

Common name	"Old" name	Current name
Side-chain cleavage enzyme; desmolase	$P450_{SCC}$	CYP11A1
3 beta-hydroxysteroid dehydrogenase	3 beta-HSD	3 beta-HSD
17 alpha-hydroxylase/17,20 lyase	$P450_{C17}$	CYP17
21-hydroxylase	$P450_{C21}$	CYP21A2
11 beta-hydroxylase	$P450_{C11}$	CYP11B1
Aldosterone synthase	$P450_{C11AS}$	CYP11B2
Aromatase	$P450_{aro}$	CYP19

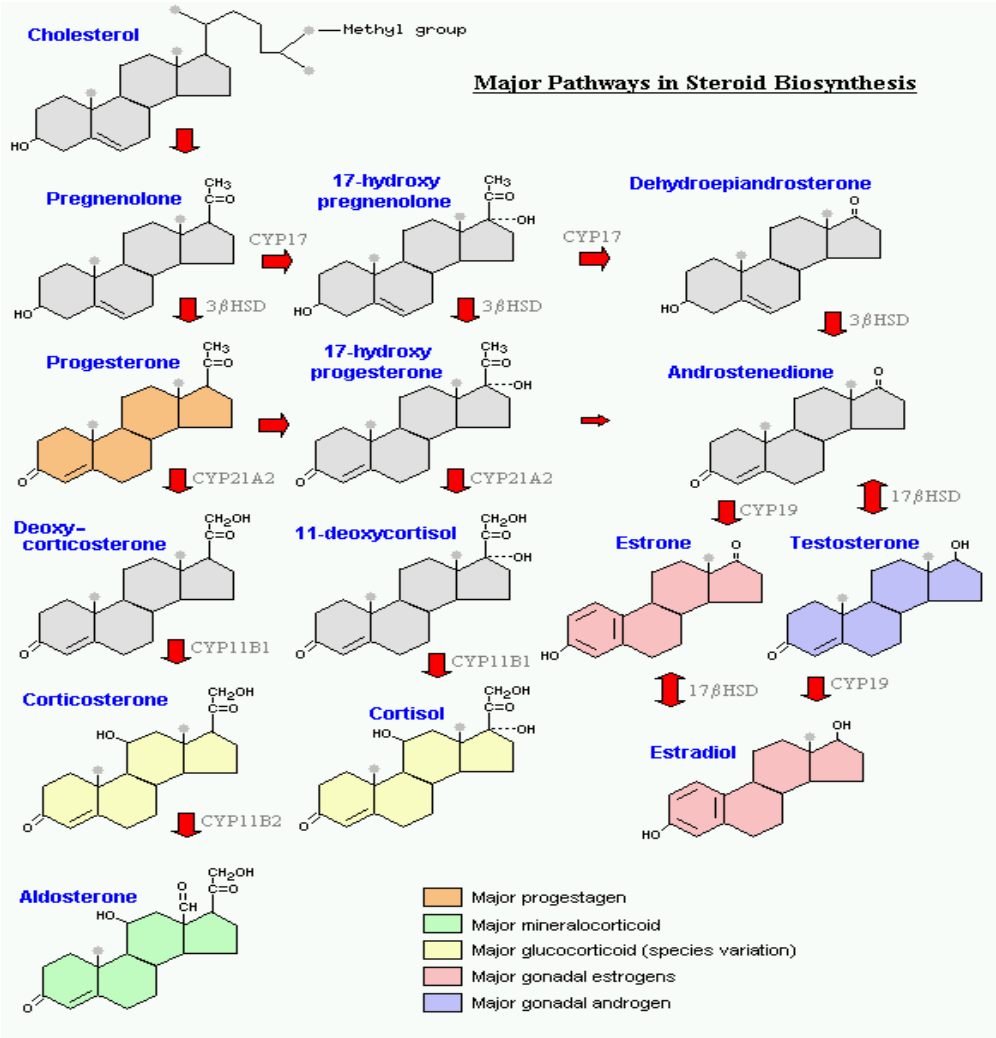


Figure 1.2 The biosynthetic pathways for major representatives of these classes of steroid hormones (Bowen, 2001).

1.3 Endocrine Disrupting Chemicals

Endocrine disrupting chemicals (EDCs) as an exogenous, have an effect on the endocrine system of animals including humans and effect in an intact organism, or its progeny, or (sub) populations (Bergman, Heindel, Jobling, Kidd & Zoeller, 2013). EDCs present in variable environmental areas such as water, air and soil. They mimic an estrogen-like response in organisms or antagonist effect can be seen. These chemicals may be natural or synthetic (Singhal et al., 2010).

EDCs are detected in food products (flax, soybeans, yams), household products (degradation products of detergents and associated surfactants, including octylphenol and nonylphenol), plants (phytoestrogens, grasses, beans, vegetables), plastics (phthalates, bisphenol A), pesticides (atrazine, DDT, nitrofen, endosulphan), pharmaceuticals (cimetidine, drug estrogens - birth control pills), industrial chemicals (benzo(a)pyrene, dioxin, PCBs), metals (cadmium, lead, mercury) (PUBH 5103, 2003a), paper production and fuel combustion. Some of the endocrine disruptive chemicals properties are shown on Table1.2 (Singhal et al., 2010).

Table 1.2 Properties of some of the endocrine disruptive chemicals (Singhal et al., 2010).

EDC	MOL. WT. (g/mol)	LOG K_{oc} (L/kg)	LOG K_{ow} (L/kg)	LOG K_d (L/kg) FOR SLUDGE		SOLUBILITY (mg/L)	pK_a
				BIOLOGICAL	DIGESTED		
Estradiol[C]	272.4	2.55–4.01	NA[D]	NA	NA	13.0–32.0	10.5–10.71
17β-Estradiol (E2)	272.4	3.10–4.01	3.9-4.0	2.4-2.8	2.3-2.5	13.0	10.71
Estrone (E1)	270.4	2.45–3.34	3.1-3.4	2.4-2.9	2.4-2.6	6.0–13.0	10.3–10.8
Ethinylestradiol (EE2)	296.4	2.91–3.04	2.8-4.2	2.5-2.8	2.3-2.6	4.8	NA
Estriol (E3)	288.4	2.13–2.62	NA	NA	NA	32	10.4
Bisphenol A (BPA)	228.0	2.50–6.60	NA	NA	NA	120–300	9.6–11.3
Nonylphenol (NP)	220.2	3.56–5.67	3.8->4.75	NA	NA	4.9–7.0	10.28
Nonylphenol ethoxylates (NP1EO-NPnEO; n ≤ 20)	264.0-1101.6	3.91–5.64	NA	NA	NA	3.02–31.9	NA
Octylphenol	206.3	3.54–5.18	NA	NA	NA	12.6	NA

Endocrine disrupting chemicals can be classified as follows;

EDCs can be categorized into the seven species (Bergman et al., 2013).

- Steroids compounds
- Phytoestrogens
- Surfactants
- Pesticides
- Polychlorinated compounds
- Organotin Compounds
- Organic oxygen compounds

One of the recent major concerns is the steroid compounds in water or wastewater. Steroid compounds are the natural and synthetic steroid hormones. Steroid hormones have lipophilic characters, because of this they tend to be transported through the blood by specific "carrier" proteins, and are able to passively enter cells and interact with receptors inside the cells (Bergman et al., 2013).

Natural estrogens, including at least six different estrogen hormones (e.g.Estrone, Estriol, 17-Estradiol), are generated by female of fertility age. Estrone, estriol and 17-estradiol are the major natural human derived estrogens. All three molecules have a 17-carbon system, which are the steroid with a methyl group at carbon-13 and an aromatic ring with a hydroxyl group at carbon-3 (Figure 1.3). The most active estrogen is 17-estradiol excreted by the ovaries, and is generated from androgens by the aromatize complex of enzymes. Synthetic estrogens are the birth control pill or Hormone Replacement Treatment for example, ethynylestradiol and diethylstilbestrol and they also found in wastewater effluent. Testosterone and progesterone can be detected in lower amounts than estrogens in wastewater. Major sources of testosterone and progesterone are food, in particular meat products. The hormones have been used as growth accelerator in the livestock. Steroid compounds almost never soluble in water because they tend to be lipophilic. Therefore steroid compounds generally adsorbed onto particles in the water or wastewater. Synthetic steroids have lower solubility than natural ones (Gill et al., 2007).

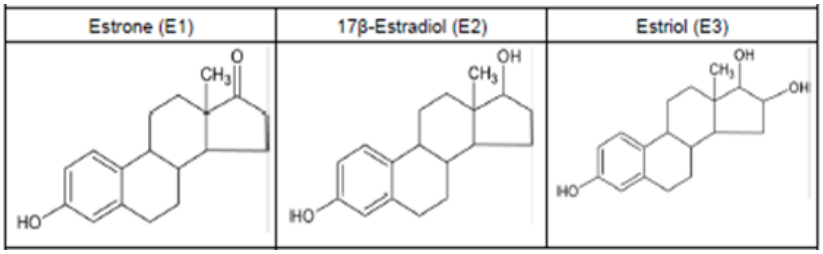


Figure 1.3 Molecular structures of estrogenic hormones.

The EDCs enter the environment from variable sources. The primary sources of EDCs in environment are wastewater treatment plants. In literature, the most common EDCs are reported as Estrone (E1), Estriol (E3), Ethinylestradiol (EE2), 17β-Estradiol (E2), BisphenolA (BPA), Nonylphenol ethoxylates (NPnEO), Octylphenol, Nonylphenol (NP). EDCs possibly cause many disorders in humans. However, no direct link has been established between EDC exposure and a human disorder, yet. On the other hand, the effects of EDCs to wildlife have been reported by laboratory studies (Singhal et al., 2010).

The concentrations of EDCs are partitioned in sediments, wastewater, air and drinking water in natural environment. Most of EDC's lipophilic and their solubility in water is low, which makes them easily adsorbed on a solid phase or associated with solid phase. Therefore, the highest concentration of EDCs could be in sediments and then in wastewater, the small amounts present in air and drinking water because EDCs have low vapor pressure (Singhal et al., 2010).

Biodegradation rates of E1, E2, E3 (the natural estrogens) and EE2 (the synthetic estrogen) are variable in wastewater treatment plant. It has been reported that they are biodegradable under aerobic condition and biodegradability degree is in the order of E3>E2>E1>EE2 (Singhal et al., 2010).

1.4 Health Effects of EDCs

EDCs are believed to have effects on some of women diseases such as endometriosis (PCBs, phthalates and dioxins) and fibroids (phthalates), early puberty

and breast development. But, there is no evidence about polycystic ovary syndrome or infertility. Few researches have reported some chemical could cause these diseases directly (Bergman et al., 2013). According to scientific studies in males, some of reproductive disease increased because of the EDCs exposure. The rate of testicular cancer has increased, semen quality has decreased and sperm counts are in the subfertile in some countries (Bergman et al., 2013). Some scientific studies in the rat indicated that there is an interrelationship between exposure to EDCs during fetal male and testicular dysgenesis (Bergman et al., 2013).

Some effects of EDCs on aquatic animals have been reported so far. The feminized male fish which exposed to estrogenic chemicals from sewage effluents have been seen in many countries. Male fish exposed to endocrine disrupting chemicals have reduced reproductive success of sperm and sperm production (Bergman et al., 2013). Some laboratory studies indicated that sex ratio is imbalanced in some animal populations such as mollusk, wild wish, and mouse when they exposed to EDCs. In the mouse model, the effects of dioxin on sex ratio are now verified (Bergman et al., 2013). Males offspring were exposed to EDCs, are reduced in humans e.g. in relation to 1,2-dibromo-3-chloropropane and dioxin).

EDCs (e.g. for PCBs, lead and methylmercury) have caused behavioural disorders such as sexually dimorphic behaviours in animals and behavioural defects in humans. In addition, there are evidences about neural development disorders in children and wildlife (Bergman et al., 2013).

The relationship between obesity and EDC exposed animals have been established by laboratory studies. Obesogens are disrupting endocrine system components such as controlling weight gain (Bergman et al., 2013).

In summary, it is well understood that EDCs can block, mimic, stimulate or inhibit production of natural hormones, and disrupt homeostasis (Bergman et al., 2013). Some of EDCs effects are shown on Table 1.3 (Singhal et al., 2010).

Table 1.3 Environmental effects linked to estrogens present in wastewater (Singhal et al., 2010).

EDC	SAMPLE SITE	SPECIES	EDC EFFECT
Mix of WW with PCB, PBDE, APEOs, pesticides (hormones not identified)	Potomac River, Washington, DC	*Micropterus dolomieu*/ small mouth bass	Intersex (oocytes in testes)
WWTP effluent – unidentified mix of compounds	United Kingdom: WWTP receiving waters (rivers)	*Rutilus rutilus*/ roach fish	Intersex (vitellogenin, ova, and tissue changes) characteristics in males
Bisphenol A	Review of several studies	Human	Prostate cancer development
Bisphenol A	Review of several lab studies	Human	Polycystic ovary syndrome, uterotrophic effects, decreased sperm, increased prolactin release
Octylphenol	Lab study	Fisher 344 and Donyru/2 rat strains	Persistent estrus
Ethinylestradiol (EE2)	Lab study	*Oryzias latipes*/ Medaka fish	Intersex in males: testes ova and abnormal tissue development
Ethinylestradiol (EE2)	Review of several studies	Human	Prostate cancer development
Nonylphenol (NP)	Lab study	Sprague-Rawley female rats	Irregular estrous cycles and advanced onset of tissue development
Nonylphenol (NP)	Lab study	Human males	Decrease in sperm production
17-Estradiol (E2)	Review of several studies	Rats	Delay in age of first estrus and vaginal opening; irregular then persistent estrus; disorders in ovarian and mammarian development
17-Estradiol (E2)	Field study	*Chrysemys pictal*/ female painted turtles	Increased E2 levels needed for vitellogenin induction of female eggs

1.5 Sources and Pathways of Hormones in Environment

Hormones enter environment through both point (e.g., effluents from WWTPs) and non-point sources (e.g., surface runoff from agriculture operations). Steroidal hormones are mainly excreted to aquatic environment from human and animals. The other source of steroidal hormones is green plant processing industry (point source) which contributes phytoestrogens in surface water bodies. After excretion, all of hormones (the natural and synthetic hormones and their metabolites) reach WWTP. Sources and pathways of hormones in environment are shown in Figure 1.4 and EDC transport pathways through different environmental media are shown in Figure 1.5 (Hamid & Eskicioglu, 2012).

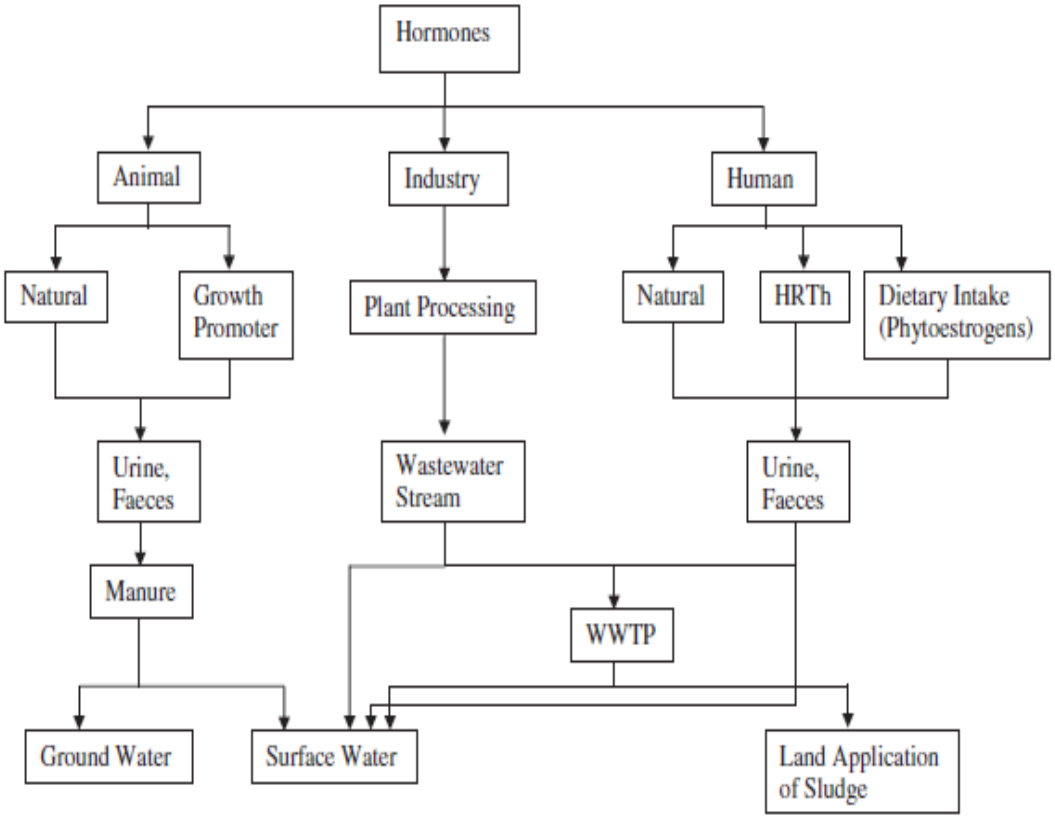


Figure 1.4 Sources and pathways of hormones in environment. HRTh: Hormone replacement therapy WWTP: Wastewater treatment plant (Hamid & Eskicioglu, 2012).

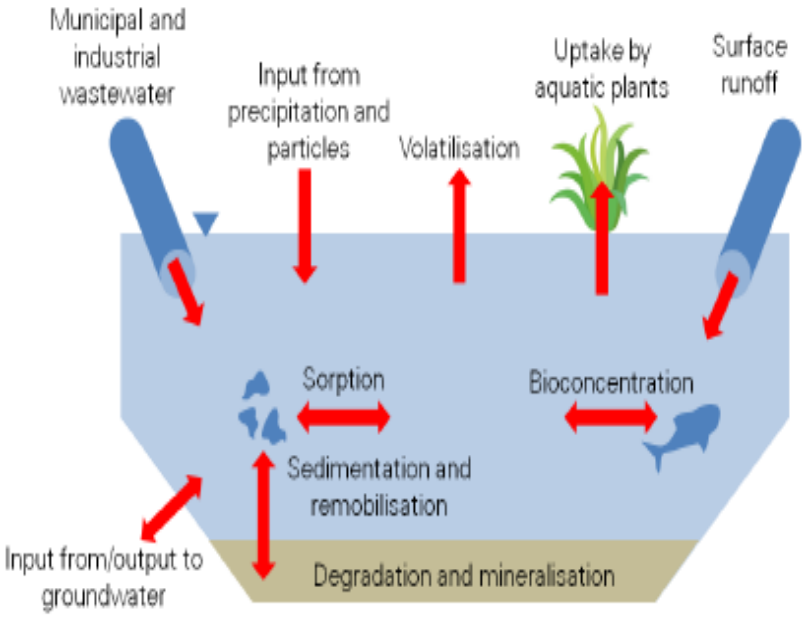


Figure 1.5 EDC transport pathways through different environmental media (Singhal et al., 2010).

1.6 Degradation of Hormones

The natural (E1, E2, E3) and synthetic estrogen (EE2) appear to be biodegradable at varying levels and the observed biodegradation order (highest to lowest) is estriol (E3) > 17β-estradiol (E2) > estrone (E1) > 17α-ethinylestradiol (EE2). E2 is oxidized E1 rapidly under aerobic conditions. In contrast, reduction of E1 to E2 under anaerobic condition could occur. But, this reaction has not been approved, yet. Degradation products of E1, E3 and EE2 are presently unknown. These transformations are shown in Figure 1.6. Besides that, microbial degradation ratio of E2 can be intercorrelated with increasing water temperature.

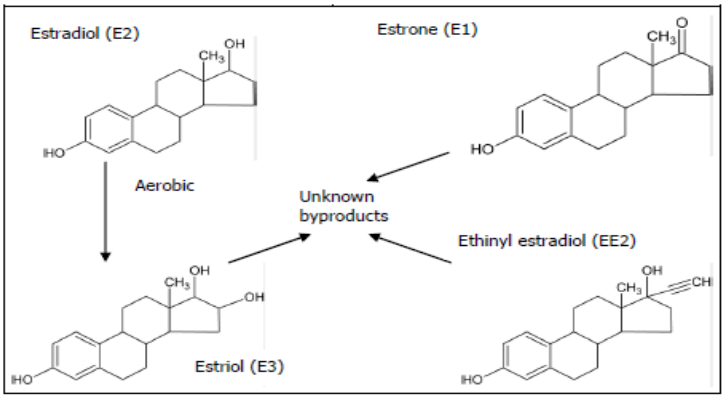


Figure 1.6 Proposed biodegradation and biotransformation mechanisms of estrogenic hormones.

1.7 Measurement Techniques of Steroidal Hormones EDCs

The toxicological studies are displayed that endocrine systems and homeostasis of animals including humans, its progeny and (sub) populations are affected by EDCs. Biological activities of EDCs are even effective in the lower range of concentrations. Because of this, the measurement of EDCs concentrations in wastewater, drinking water, surface water and ground water is very important.

The establishment of method to get real data for the concentration of any EDC is challenging because of: (1) the large number and chemical diversity of the compounds of interest; (2) the need to quantify low levels (ng/ L or pg/L) in an organic matrix; and (3) the complexity of sample concentration techniques. Therefore, a considerable effort should be given to the analytical methods for the measurement of EDCs. Steroid hormones are just one of these EDCs and they are major interest as the other synthetic EDCs. Chromatographic techniques (e.g. GC-MS/MS, LC-MS/MS) and non-cellular assays can be used for determination of different EDCs from different environmental samples. Enzyme linked immunosorbent assay (ELISA) is a widely used non-cellular assay that is available for specific EDCs (Belfroid et al., 1999). Although quantification of the EDCs can be performed rapidly by ELISA methods with low-cost, different responses can be achieved by different ELISA kits and the cross-reactivity can be performed between the different estrogens exist in the sample. GC-MS, GC-MS/MS or LC-MS, LC-MS/MS are the high technology instruments used for analysis of organic substances as well as estrogens (Singhal et al., 2010). These instruments are more reliable than spectrophotometric or immunoassay methods. Therefore, gas and liquid chromatographic techniques have been used for determination of EDCs hormones. The most common problem associated with measurement of estrogenic hormones in GC or GC-MS/MS derivatization of the samples. Endocrine disrupting hormones are non-volatile compounds and they need derivatization to be measured. Various derivatization techniques can be applied. However, derivatization procedures are long and its reproducibility could be low. Due to these problems in GC, liquid chromatographic techniques in estrogenic hormone measurement are preferred.

1.8 Literature Review

Many studies were performed by scientists about determination of EDCs in water samples. One of those studies was carried out by Isobe et al. in 2003. An analytical method was developed and applied for analysis of steroid estrogens and their conjugates in water samples. Samples were collected from lake, river and effluent of WWTP in Japan. The water samples were stored in amber glass bottles and transported in cold. 1L sample was filtered through glass fiber filter and then acidified to pH 3.5-5 with acetic acid. Samples were preconcentrated, purified and fractioned with SPE. Eluates were evaporated by gentle nitrogen gas and then dissolved in 100 µL–1 mL of 5% acetonitrile /H_2O (v/v). Instrumental analysis was performed with LC-MS/MS (CapLC (Waters, USA) liquid chromatograph equipped with a Quattro (Micromass, UK) tandem mass spectrometer.) Chromatographic separation was performed with Zorbax Extend-C18 column (150 mm ×1 mm I.D 3.5 µm, Agilent). The column was kept at 30 ºC, mobile phase flow rate was 40 µL/min and injection volume was set at 10 µL. Mobile phase gradient conditions were shown in Table 1.4.

Table 1.4 Mobile phase gradient conditions reported by Isobe (2003).

Acetonitrile (%)	Water (%)	100 mM Triethylamine in Water (%)	Time (min)
0	80	20	0
40	40	20	12
80	0	20	15
0	0	20	17

The mass spectrometer was operated in negative mode electrospray ionization (ESI (-)) in Multiple Reaction Monitoring (MRM) mode and conditions were as follows; flow: 70 L/h; desolvation gas flow: 500 L/h; 2.7 kV; multiplier voltage: 650 V. Four HPLC columns were compared for chromatographic separation and responses of analytes. The columns used were TRP-100 (150 mm×1 mm I.D., 5 µm, Supelco), Asahipak ODP-40 (150 mm×1 mm I.D., 4 µm, Showa Denko K.K.), Zorbax Extend-C_{18} (150 mm×1 mm I.D., 3.5 µm, Agilent) and XTerraMS C_{18} (150

mm×1 mm I.D., 3.5 µm, Waters). Zorbax Extend-C_{18} and XTerraMS C_{18} are provided better chromatographic separation of the target analytes due to smaller particle size (3.5µm). Triethylamine (TEA) and ammonia were used to increase efficiency of ionisation of estrogenic hormones. The performance of the analytical method with Zorbax Extend-C_{18} by using acetonitrile as mobile phase was depicted in Table 1.5.

Table 1.5 Validation results of method developed by Isobe (2003).

Analytes	Sample from	Sample volume(mL)	% Recovery (10 ng of std was spiked to 1 liter)	LOD (ng/L) (from S/N= 3)	Linear Working Range(ng/L)
E1 E2 E3 EE2	Lake Water (Kasumigaura)	1000	116 92 81 90	0.1 0.3 1.5 0.2	500–100000
E1 E2 E3 EE2	Milli-Q water	1000	101 76 80 82	- - - -	500–100000

Laganà et al. (2004) studied on analysis of estrogenic hormones in different matrix as surface water and STP influent, effluent. The samples were collected from Italian river and municipal sewage treatment plants (influent and effluent water) located in Rome, respectively. The samples were preserved at 4°C and extraction was carried out within 24–36 h. The solid phase extraction method was used in this study. Shortly, analytes were extracted and concentrated using Oasis HLB SPE analytical cartridge. 100 mL of influent, 250 mL effluent (STP) and 1000 mL of river water were extracted. After extraction procedure, extracts were concentrated to dryness under gentle N_2 stream. Finally, analytes were redissolved in 0.2 mL H_2O: ACN (50:50). 50µL of sample was injected to LC-MS/MS ESI system (Perkin-Elmer binary LC pump Series 200 (Perkin-Elmer, Norwalk, CT) equipped with a Rheodyne 7125 injector with a 50 µL Loop). The analytes were chromatographed on a 25 cm × 4.6mm i.d. column filled with 5 µm (average particle size) LC-18 packing Alltima (Alltech, Deerfield, IL). The flow rate of mobile phase was 1 mL/min and 200 µL of the column effluent was diverted into the ESI source. Post-column addition (before

splitting) of 0.11 ml/min of ammonia solution (50 mmol) was performed. The MS/MS conditions were optimized for sensitivity and selectivity. Analysis was conducted in the negative ionization mode. Source temperature was 350 ◦C. Multiple reaction monitoring (MRM) experiments were done to detect ion transitions. Acetonitrile was taken as a mobile phase component as shown in Table 1.6. The chromatographic separation of the method was given in Figure 1.7. Method performance and validation results were presented in Table 1.7.

Table 1.6 Mobile phase gradient conditions reported by Laganà (2004).

Acetonitrile (%)	Water (%)	Time (min)
40	60	0.00
40	60	5.00
75	15	20.00
95	5	20.01
100	0	30.00
100	0	35.00

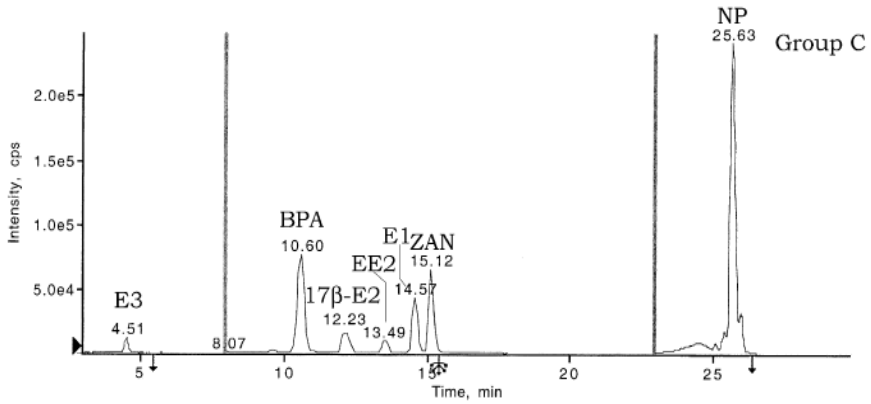


Figure 1.7 Chromatographic separation of analysis (Laganà, 2004).

Table 1.7 Validation results of method developed by Laganà (2004).

Analytes	Sample from	Sample volume(mL)	% Recovery	LOD (ng/L) (from S/N= 3)	Linear Working Range(ng/L)
E1 E2 E3 EE2	Influent of STP	100	95 95 91 96	1.2 1.9 7.0 1.6	–
E1 E2 E3 EE2	Effluent of STP	250	89 95 92 96	0.8 0.8 0.5 1.1	–
E1 E2 E3 EE2	River Water	1000	99 96 97 100	0.1 0.2 0.3 0.4	–

Another study about estrogenic hormone measurement was performed by Vanderford et al. (Vanderford, Pearson, Rexing & Snyder, 2003). Water samples were collected from Boulder Basin of Lake Mead. They were kept in 1L amber glass bottle at 4°C, pH was adjusted to 2.0 with concentrated sulphuric acid and extracted within 14 days. In sample preparation step, sample was extracted and purified by SPE techniques (HLB cartridges from Waters Corp). First, SPE cartridges were preconditioned with 5 mL of methyl tert-butyl ether (MTBE), 5 mL of methanol, and 5 mL of reagent water and then dried with gentle nitrogen stream. After this process, samples were loaded into SPE and eluated with 5 mL of 10/90 (v/v) methanol/MTBE followed by 5 mL of methanol. Eluates were concentrated to 1mL. After that, 10µL of sample injected to LC-MS/MS (An Agilent (Palo Alto, CA) G1312A binary pump and an HTC-PAL auto sampler (CTC Analytics, Zwingen, Switzerland)) equipped with API 4000 triple quadrupole mass spectrometer (APCI ion source positive mode). Synergi Max-RP C_{12} (250×4.6mm, 4 µm) column was used. Methanol and 0.1% formic acid (v/v) in water were used as mobile phase component. Gradient method was performed (Table 1.8) and the analysis time was more than 30 minutes. Flow rate of mobile phase was set to 700 µL/min. The performance of the analytical

method by using methanol as mobile phase was depicted in Table 1.9. Methanol and acetonitrile are the most widely used mobile phase components for chromatographic separation of estrogenic hormones. Methanol and acetonitrile have moderate polarity and a good chromatographic separation was obtained by this method.

Table 1.8 Mobile phase gradient conditions reported by Vanderford (2003).

Methanol (%)	0.1% Formic acid (v/v) in water (%)	Time (min)
5	95	0.00
5	95	3.50
80	20	10.00
80	20	13.00
100	0	13.01
100	0	21.00
5	95	21.01
5	95	30.00

Table 1.9 Validation results of method developed by Vanderford (2003).

Analytes	Sample from	Sample volume(mL)	% Recovery (spiked 10 ng/L)	LOD (ng/L)	Linear Working Range(ng/L)
E2 EE2	Surface water	1000	92 96	1 1	1 to 100

Rodriguez et al. (Rodriguez, López de Alda & Barceló, 2004) studied on method development for measurement of estrogenic hormones at ng/L concentration. The study was performed by HP 1100 auto sampler HPLC system (Agilent Technologies) equipped with a HP 1090A LC pump and Electro Spray Ionization (ESI) mass spectrometer in negative mode (LC-ESI-MS). SPE (LiChrolut RP-18 cartridges 500mg) extraction procedure was applied. Volume of samples that were collected ground water from Llobregat River in Barcelona and drinking water from drinking water treatment plant were 500 mL. The final volumes of the extracts were 300 µL with methanol after sample preparation. The injection volume to LC-MS/MS system were fixed to 20µL. Chromatographic separation was carried out with LiChrospher 100RP-18 (250mm × 4 mm, 5µm particle diameter) column. Mobile phase

components consisted of acetonitrile and pure water. Flow rate of mobile phase was fixed to 1mL/min. Mobile phase gradient conditions was given in Table 1.10. The performance of the analytical method by using acetonitrile as mobile phase was depicted in Table 1.11.

Table 1.10 Mobile phase gradient conditions reported by Rodriguez (2004).

Acetonitrile (%)	Pure water (%)	Time (min)
10	90	0.00
100	0	30.00
10	90	30.01
10	90	40.00

Table 1.11 Validation results of method developed by Rodriguez (2004).

Analytes	Sample from	Sample volume (mL)	% Recovery	LOD (ng/L)	Linear working range(µg/L)
E1 E2 E3 EE2	Water sample	500	100 98 94 91	2.5 2.5 5.04 3.22	5–1000

Trenholm et al. (2006) studied on analysis of estrogen hormone in different matrix as wastewater influent and effluent samples (Trenholm, Vanderford, Holady, Rexing & Snyder, 2006). Samples were collected in 1L amber glass bottle and preserved at 4°C, at pH=2 (H_2SO_4). Analytes are extracted and concentrated using a 500 mg Oasis HLB SPE analytical cartridge. After extraction procedure, extracts were concentrated to 1mL under gentle N_2 stream. Finally, 10µL of sample was injected to LC-MS/MS ESI (Agilent G1312A, Palo Alto, CA) and an auto sampler (HTC-PAL, CTC Analytics, Zwingen, Switzerland) system. Synergi Max-RP C12 (25.0cm×0.46 cm) with 4µm particle size chromatographic column was used. The flow rate of mobile phase was 700 µL/min. The MS/MS conditions were optimized for sensitivity and selectivity**.** Selected reaction monitoring (SRM) experiments were done to detect ion transitions. 0.1% formic acid (v/v) in water and methanol were used as mobile phase components. The condition of mobile phase gradient was shown in Table 1.12

and the results of the study with methanol as mobile phase were summarized in Table 1.13.

Table 1.12 Mobile phase gradient conditions reported by Trenholm (2006).

Methanol (%)	0.1% Formic acid (v/v) in water (%)	Time (min)
5	95	0.00
5	95	3.50
80	20	10.00
80	20	13.00
100	0	13.01
100	0	21.00
5	95	21.01
5	95	30.00

Table 1.13 Validation results of method developed by Trenholm (2006).

Analytes	Sample from	Sample volume (mL)	% Recovery	LOD (ng/L)	Linear working range(µg/L)
E1 E2 E3 EE2	Water sample	500	100 98 94 91	0.534 0.297 0.587 0.256	5–1000

Farr´e et al. (2007) conducted method development for estrogenic hormone determination in different matrixes as surface water and wastewater. Natural water, river water and WWTP water were collected. 1 L samples was filtered (0.45µm HVLP filters) and stored at 4°C in the dark. In sample preparation step, sample was extracted and purified by SPE techniques. Afterwards, reconstituted in 0.5mL methanol and then injected (25µL of sample) to LC-MS/MS (Waters Alliance 2690 LC and Quattro LC triple-quadrupole mass spectrometer). Purospher STAR-RP-18 (125×2.0mm, 5 µm) column was used. LC-MS/MS interface ionization was carried out at ESI in negative ionization mode Acetonitrile and deionized water was the mobile phase. Gradient method was performed (Table 1.14) and flow rate of the mobile phase was fixed at 200 µL. Purospher STAR-RP-18 (125×2.0mm, 5 µm)

column and Waters Acquity C_{18} (50×2.1 mm, 1.7 µm) column were compared. The results indicated that chromatographic separation performed with smaller particle size column (Waters Acquity C_{18} with 1.7 µm particle size) provided relatively faster analysis and less solvent consumption. The performance of the analytical method by using acetonitrile as mobile phase and Waters Acquity C_{18} column was depicted in Table 1.15. LC-MS/MS chromatogram obtained by this method was shown in Figure 1.8.

Table 1.14 Mobile phase gradient conditions reported by Farr´e (2007).

Acetonitrile (%)	Water (%)	Time (min)
10	90	0.00
50	50	5.00
80	20	25.00
100	0	25.01
100	0	29.00
10	90	31.00
10	90	44.00

Table 1.15 Validation results of method developed by Farr´e (2007).

Analytes	Sample from	Sample volume (mL)	% Recovery	LOD (ng/L)
E1 E2 E3 EE2	Water sample	500	100 98 94 91	0.4 0.5 2 1

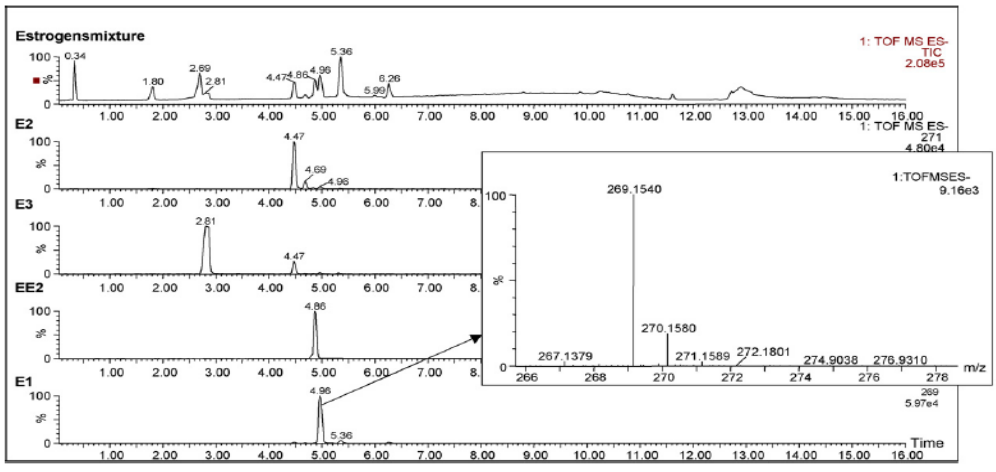


Figure 1.8 Total ion chromatogram for standard solutions of estrogens obtained from method developed by Farr´e (2007).

Another method development study was conducted at ng/L estrogenic hormone concentration in year 2008 by Kuster et al. Water samples were collected from Llobregat River, WWTP effluent and drinking water. It was reported that various sewage treatment plant effluents are connected to Llobregat river basin. Estrogens were extracted (20 mL of samples) with PLRP-s (cross linked styrene divinylbenzene). First, samples were loaded into SPE cartridges by automated solid-phase extraction system. After that, SPE cartridges were washed and eluted directly onto chromatographic column by mobile phase flow. Electrospray ionization mode was performed in negative mode. Auto sampler injection volume was fixed to 20 µL. Chromatographic separation was carried out with Purospher STAR-RP-18 (125×2.0mm, 5µm) column. Mobile phase components consisted of acetonitrile and pure water. Flow rate of mobile phase was fixed to 0.2 mL/min. Mobile phase gradient conditions and method performances were depicted in Table 1.16 and Table 1.17, respectively.

Table 1.16 Mobile phase gradient conditions reported by Kuster (2008).

Acetonitrile (%)	Pure water (%)	Time (min)
10	90	0.00
50	50	5.00
80	20	25.00
100	0	25.01
100	0	30.00
10	90	32.00
10	90	45.00

Table 1.17 Validation results of method developed by Kuster (2008).

Analytes	Sample from	Sample volume (mL)	% Recovery	LOD (ng/L)
E1	Water sample	500	99	0.24
E2			85	0.85
E3			70	0.62
EE2			89	0.62

Analysis of estrogenic hormone in different matrix as surface water and wastewater was conducted by Pedrouzo et al. (2009) (Pedrouzo, Borrull, Pocurull &

Marcé, 2009). The samples were collected from Ebro river and Catalan domestic sewage treatment plants (influent and effluent water) Spain. They were preserved at 4°C and at pH=2 (HCl). Analytes were extracted and concentrated using a 12mL, 500 mg Oasis HLB SPE analytical cartridge. 100 mL of influent, 250 mL effluent (STP) and 500 mL of river water were extracted. After extraction, extracts were concentrated to dryness under gentle N_2 stream. Finally, analytes were redissolved with 1mL H_2O: MeOH (20:80). 50µL of sample was injected to LC-MS/MS ESI system. Kromasil 100 C18 (25.0cm×0.46 cm) with a 5µm particle size was used. The flow-rate of mobile phase was 1 mL/min and the column temperature was kept at 35°C. The MS/MS conditions were optimized for sensitivity and selectivity. Spray potential was 3000 V, a nebulizer was 45 psi and a source temperature was 350 ◦C and drying gas flow was 12 L/min. Selected reaction monitoring (SRM) experiments were done to detect ion transitions. Acetonitrile and methanol were tested as mobile phase for chromatographic peak shape and resolution of analytes. Acetonitrile resulted in better peak separation compared to methanol. Addition of acetic acid (pH 2.8) improved peak resolution. The mobile phase was selected as acetonitrile, Milli-Q water with acetic acid. Gradient method was performed. The analysis time was nearly 35 minutes. The results of method validation were summarized in Table 1.18.

Table 1.18 Validation results of method developed by Pedrouzo (2009).

Analytes	Sample from	Sample volume(mL)	% Recovery	LOD (ng/L) (from S/N= 3)	Linear Working Range(ng/L)
E1 E2 E3 EE2	Influent of STP	100	60 53 56 37	50 100 50 100	150–1000
E1 E2 E3 EE2	Effluent of STP	250	51 61 59 52	10-35 70 10-35 70	100–1500
E1 E2 E3 EE2	River Water	500	49 32 49 68	15 30 30 30	75–1500

The extraction method is another question to obtain low detection limits. Sun et al. (Sun, Yong, Chu & Lina, 2009) studied on sample extraction and concentration techniques by using different SPE (Supelco). SPE cartridge and Solid Phase Disk Extraction (SPDE) (ENVI-18 SPE disk from Supelco) techniques were applied for extraction of analytes and compared for extraction efficiencies. 100 mL deionized water, tap water and waste water samples were collected and stored at 4°C in the dark. In sample preparation step, sample was extracted and purified by SPE or SPDE. Afterwards, they were dissolved in 0.1mL acetonitrile and then injected (5µL of sample) to LC-MS/MS. Acquity UPLC BEH RP-C18 (50×2.1mm, 1.7 µm) column was used. Methanol and deionized water were the mobile phase to separate target estrogens. Gradient method was performed and the analysis time was more than 10 minutes. The performance of the analytical method by using methanol as mobile phase was depicted in Table 1.19 with SPE techniques.

Table 1.19 Validation results of method developed by Sun (2009).

Analytes	Sample from	Sample volume(mL)	% Recovery (spiked 10 ng/L)	LOD (ng/L) (from S/N= 3)	Linear Working Range(ng/L)
E1 E2 E3 EE2	Deionized water	100	88.0 93.2 88.4 90.6	–	1 to 50
E1 E2 E3 EE2	Tap water	100	82.2 92.9 90.4 86.9	–	1 to 50
E1 E2 E3 EE2	Waste water	100	83.0 91.8 86.7 88.8	0.5 0.6 1 1.2	1 to 50

A study for measurement of sub-ng/L level of estrogens was performed by Vulliet et al. (2008) (Vulliet, Wiest, Baudot, Florence & Loustalot, 2008). HP1100 HPLC system (Agilent Technologies) equipped with a triple quadrupole mass spectrometer

(Applied Biosystem/3200 QTrap) was used. SPE (Strata C18-E 200mg and the surface modified styrenedivinylbenzene Strata X 200mg from Phenomenex) extraction procedure was applied. Volume of samples extracted was 1 L. The final volumes of the extracts were 1mL after sample preparation. The injection volume to LC-MS/MS system were fixed to 100µL. Chromatographic separation was carried out by Zorbax Eclipse XDB C18 (100mm×2.1mm, 3.5 µm) column. Mobile phase components consisted of acetonitrile and pure water. The resulting detection limits were considerably low compared to the other studies. The column used in this study might be the reason for low LOD values. On the other hand, although the method detection limits range 0.008 µg/L to 0.02 µg/L, the linear working range was 50 ng/L to 2000 ng/L as shown in Table 1.20. This method is very sensitive for detection of very low concentrations. However, the measuring range (linear working range) is not consistent with these low LOD values. The performance of the analytical method by using acetonitrile as mobile phase was depicted in Table 1.20.

Table 1.20 Validation results of method developed by Vulliet (2008).

Analytes	Sample from	Sample volume (mL)	% Recovery (pH=3)	Instrumental detection limit (µg/L) (from S/N= 3)	Method detection limit(ng/L) (from S/N= 3)	Linear working range(µg/L)
E1	Water sample	1000	97.5	0.02	0.02	0.05–20
E2			98.8	0.008	0.01	
E3			96.9	0.02	0.03	
EE2			96.8	0.18	0.20	

The studies showed that reverse phase chromatographic separations were performed commonly for analysis of estrogenic hormones. Effective surface area of stationary phase, mobile phase composition and flow rate are most important factors for chromatographic separation. In order to achieve low detection limits, ionization efficiencies of target analytes should be advanced and interferences should be removed from samples prior to injection.

1.9 Objectives and Scope

EDCs are complex chemical. Their effect on human and animals are not well known yet. But, it could be a main concern to protect human and animal life soon when their effects are clearly understood. It is evident that their concentration in the water is very low. Therefore, it is not easy to detect their existence in water. There is an urgent need to develop methods to be able to measure very low concentrations of these chemicals. Measuring very low concentrations at ng/ or pg/L levels will help to understand adverse effect of these chemicals.

The objective of this thesis is to develop an instrumental method for the measurement of estrogenic hormones as E1, E2, E3 and EE2 in LC-MS/MS. The scope is to determine the optimum operation conditions of LC-MS/MS to obtain the lowest LOD.

In this scope, the following studies were conducted.

- Determination of optimum mobile phase composition
- Investigation of effect of alkaline mobile phase on ionization of hormones
- Optimization of ionization conditions
- Development of calibration curve for the determined optimum instrument operation conditions.

CHAPTER TWO
MATERIALS AND METHODS

2.1 Chemicals

All the reagents used were analytical grade. Estrogenic hormones (E1, E2 and EE2, E3) were bought from Sigma–Aldrich (St Quentin Fallavier, France). The purity of hormones was at least 98%. Stock solutions of hormones were 10 mg/L in methanol. Working solutions to inject LC-MS/MS were prepared by diluting appropriate parts of each individual solutions in acetonitrile/water (28/72, v/v). HPLC grade acetonitrile and ammonia solution (25% in water) were bought from Merck. Milli-Q Pure water (18.2 M Ω cm^{-1}) was set in preparation of mobile phase and solutions.

2.2 Instrument

Thermo TSQ Quantum Access Max Triple Quadropole MS liquid chromatography system (with an auto sampler, a degasser, a column oven, and a binary pump) was used in this study. X-calibur software from Thermo was used to data processing. Chromatographic separation was performed with Hypersil gold analytical column C18 / (2.1mm x 50mm / 1.9μm Particle Size). Auto sampler temperature and column oven temperature were fixed at 25^0C and injection volume was set to 25 μL in fool loop mode. Electro Spray Ionization (ESI (-)) technique was performed in negative mode and SRM mode (selected reaction monitoring) was used to analyses hormones. Isocratic elution program was applied. Precursor (Parent mass) and product ion masses as well as the individual declustering potential and collision energy voltages of each hormone are shown in Table 2.1.

Table 2.1 SRM mode conditions of the MS/MS detector.

Name		**Parent Mass**	**Product Mass**	**SRM Collision Energy**	**Tube Lens**	**Polarity**
E1	1	269.1	143.2	51	85	-
E1	2	269.1	145.3	38	85	-
E2	3	271.1	145.1	43	85	-
E2	4	271.1	183.2	41	85	-
E3	5	287.0	145.0	37	85	-
E3	6	287.0	171.1	38	85	-
EE2	7	295.1	145.0	45	85	-
EE2	8	295.1	159.1	37	85	-

2.3 Experimental Conditions

The study contains six parts as follows; 1) pre-optimization of mobile phase, injection solution compositions and flow for peak symmetry and resolution factor by using central composite design, 2) improvement of integration area with the addition of alkaline solution into mobile phase, 3) determination of ranges of operation parameters of LC-MS/MS 4) improvement of optimization conditions for LC Elution for determined ranges of operation parameters of LC-MS/MS by using Box Behnken response surface method, 5) further improvement of optimized conditions for LC-MS/MS operation, 6) development of calibration curve for the optimized instrumental and elution conditions. The experimental conditions of each study were given in detail in Results and Discussion Chapter.

CHAPTER THREE
RESULTS AND DISCUSSIONS

3.1 Pre-Optimization of Mobile Phase, Injection Solution Compositions and Flow for Peak Symmetry and Resolution Factor by Central Composite Design

Optimization studies are generally conducted by using response surface methods (RSM). RSM is a kind of mathematical and statistical technique for designing experiments, building models, evaluating the relative significance of several independent variables, and determining the optimum conditions for desired responses (Box, 1978; Draper, 1988; Zhang, 2009). The two most common designs extensively used in RSM are the central composite design (CCD) and the Box-Behnken design (BBD). The CCD is ideal for sequential experimentation and allows a reasonable amount of information for testing lack of fit while not involving an unusually large number of design points (Montgomery, 1996; Myers, 1971; Somayajula, 2011). CCD is also useful for building a second order (quadratic) model for the response variable without needing to use a complete three-level factorial experiment. Coded variables are often used when constructing the design. After the designed experiment is performed, coefficients of response equation either linear or quadratic are determined by regression analysis with respect to observed responses. The final response equation with coefficients can be used predict the responses for different levels of factors.

The aim of this study was to optimize flow rate through the column, composition of mobile phase and standard solution for the best peak symmetry and resolution factor in LC-MS/MS. In other words, the effect of these three factors on the peak structure was investigated. Central composite design (CCD) was used for optimization purpose. Mobile phase and standard solution compositions were selected as acetonitrile (ACN) and water. Three factors in the design were X_1: percentage of acetonitrile in mobile phase (% ACN_m), X_2: percentage of acetonitrile in standard solution (% ACN_s); and X_3: flow rate of mobile phase through column (µL/min). The responses were peak symmetry and resolution factor for four different

estrogenic hormones as E1, E2, E3 and EE2. Table 3.1 indicates factors and their low and high values used in CCD. Coded and actual experimental points were depicted in Table 3.2. The experiments were conducted randomly and number of replicate at center point was 5. The concentration of hormones in the standard solution was 50 µg/L in mix.

Table 3.1 Factors and ranges of CCD experimental design.

Name	Units	Type	Low Actual	High Actual
Mobile Phase	%ACN	Numeric	40	70
Standard Solution	%ACN	Numeric	30	80
Flow	µL/min	Numeric	150	400

Table 3.2 CCD coded and actual experimental points.

Run Number	Coded variables			Actual Variables		
STD	X1	X2	X3	% ACN_m	%ACN_s	Flow Rate µL/min
1	0	0	-α	55.00	55.00	64.78
2	-1	-1	+1	40.00	30.00	150.00
3	+1	-1	-1	70.00	30.00	150.00
4	-1	+1	-1	40.00	80.00	150.00
5	+1	+1	-1	70.00	80.00	150.00
6	0	-α	0	55.00	12.96	275.00
7	-α	0	0	29.77	55.00	275.00
8	+α	0	0	80.23	55.00	275.00
9	0	+α	0	55.00	97.04	275.00
10	-1	-1	+1	40.00	30.00	400.00
11	+1	-1	+1	70.00	30.00	400.00
12	-1	+1	+1	40.00	80.00	400.00
13	+1	+1	+1	70.00	80.00	400.00
14	0	0	+α	55.00	55.00	485.22
15-1	0	0	0	55.00	55.00	275.00
15-2	0	0	0	55.00	55.00	275.00
15-3	0	0	0	55.00	55.00	275.00
15-4	0	0	0	55.00	55.00	275.00
15-5	0	0	0	55.00	55.00	275.00

The experiments were conducted at isocratic conditions, no gradient was applied. The auto sampler, parent and product masses, LC-MS/MS running and selected reaction mode (SRM) conditions were given in Table 3.3, Table 3.4, Table 3.5, Table 3.6, respectively. These are not the optimum conditions in operation of LC-MS/MS for studied hormones. Further studies were carried out for optimization of LC-MS/MS operation conditions.

Table 3.3 Auto sampler operation conditions for optimization of mobile phase, standard solution compositions and flow rate.

Injection Volume (µL)	25
Needle Height From Bottom (mm)	0.1
Syringe Speed (µL/s)	5
Flush Volume (µL)	400
Flush/Wash Source	Wash Bottle
Wash Volume (µL)	3000
Flush Speed (µL/s)	100
Post-Injection Valve Switch Time (min)	0
Injection Mode	Full Loop
Tray Temperature (ºC)	25
Column Oven Temperature (ºC)	25

Table 3.4 Parent and product masses and running conditions in LC-MS/MS.

	Parent Mass	Product Mass	SRM Collision Energy	Retention Time	Time Window	Tube Lens	Polarity	Trigger	Name
1	269.1	143.2	51	7.50	15.00	85	-	0	E1
2	269.1	145.3	38	7.50	15.00	85	-	0	E1
3	271.1	145.1	43	7.50	15.00	85	-	0	E2
4	271.1	183.2	41	7.50	15.00	85	-	0	E2
5	287.0	145.0	37	7.50	15.00	85	-	0	E3
6	287.0	171.1	38	7.50	15.00	85	-	0	E3
7	295.1	145.0	45	7.50	15.00	85	-	0	EE2
8	295.1	159.1	37	7.50	15.00	85	-	0	EE2

Table 3.5 Interface conditions in LC-MS/MS for optimization of mobile phase, standard solution compositions and flow rate.

Capillary Temperature (^{o}C)	280
Vaporizer Temperature (^{o}C)	120
Sheath Gas Pressure (Arb)	25
Aux Gas Pressure (Arb *)	20
Ion Sweep Gas Pressure (Arb)	2.0
Spray Voltage (V)(positive/negative polarity)	2800/3500

**Arb: Arbitrary units*

Table 3.6 The conditions of selected reaction mode (SRM) for optimization of mobile phase, standard solution compositions and flow rate.

MS Acquire Time (min)	15
Collision Gas Pressure (m Torr)	1.5
Cycle Time (s)	0.5

System was optimized to obtain a good peak shape. Good peak shape can be defined as a symmetrical or gaussian peak and poor peak shape can include both peak fronting and tailing. Good peak shape can be defined by tailing factor of 1.0, high efficiency, narrow peak width. It is important for improved resolution (Rs), more accurate quantitation and longer usable column lifetime.

Asymmetrical peaks are said to be either front or tail. Peak fronting or tailing can be caused by poor quality or polluted columns or by the dead volume of the system. Asymmetry can degrade the quality of a separation. The extent of asymmetry is expressed by either the asymmetry factory (As), or the tailing factor (Tusp or TF). In regard to the peak asymmetry, an asymmetry factor close to AS = 1 is ideal. A typical acceptable range could be $0.8 < AS < 1.8$ when working towards a reduced plate height of $h \leq 3$.

Symmetry factor (tailing factor, A_s)

The symmetry factor for a peak can be calculated using the following equation:

$$A_s = \frac{W_x}{2d} \tag{3.1}$$

Where,

W_x = peak width at 5% of peak height, measured from the baseline

d = baseline distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at 5% of the peak height, measured in the same units as W_x.

Asymmetry factor of 1.0 signifies complete symmetry. Values of A_s which are greater than 2 may lead to incorrect integration, resulting in erroneous quantitation. The main factors that influence peak symmetry depend upon retention, solvent effects, incompatibility of the solute with the mobile phase or development of an excessive void at the inlet of the column.

Resolution is a measurement used to quantify peak spacing in a liquid chromatography (LC) separation. Although very simple at first examination, resolution can be affected significantly by peak sizes and shapes.

The most common equation used for measuring resolution (Rs) is

$$R_s = 2(t_2 - t_1) / (w_1 + w_2) \tag{3.2}$$

Where,

t_1 and t_2 = retention times or baseline distances between the point of injection and the perpendicular dropped from the maximum of each of the two peaks. $t_2 > t_1$

W_1 and W_2 = the respective peak widths measured from the baseline at 5% of peak height, measured in the same units as t_1 and t_2.

The value of Rs which corresponds to a baseline separation between two symmetric peaks should be greater or equal than 1.5.

Where,

t_1 and t_2 are the retention times of the two peaks of interest, and W1 and W2 are the peak widths measured at the baseline between tangents drawn to the peak sides. From a practical standpoint, it is much easier to measure the peak width at half the peak height (Dolan, 2002).

The resolution equation using the half-height method is

$$R_s = \frac{2\left(t_2 - t_1\right)}{1.7\left(w_{0.5,1} + w_{0.5,2}\right)} \tag{3.3}$$

$W_{0.5,1}$ and $W_{0.5,2}$ are the peak widths measured at half height. The half-height method for measuring resolution is used commonly by data systems because it is much easier to measure the half-height width than the baseline width. This technique is also easier to apply to peaks that are not baseline-resolved. If the peaks are not separated fully, it can be difficult or impossible to measure the baseline width accurately. Equations 1 and 2 will give the same value of Rs if the peaks are symmetric. The valley between two symmetric peaks just touches the baseline when R_s = 1.5. Because it is a good idea to have a little extra baseline between peaks to tolerate (Dolan, 2002).

These two methods to calculate Rs could be used for symmetrical peaks. At the beginning of experiments in this study, asymmetrical peak shapes were obtained. Therefore, Rs was calculated with following equation.

$$Rs = \frac{t_2 - t_1}{d_2 - d_1} \quad (3.4)$$

Where,

t_2: retention time of following peak

t_1: retention time of leading peak

d_2: baseline distance between the perpendicular dropped from the peak maximum and the leading edge of the following peak

d_1: baseline distance between the perpendicular dropped from the peak maximum and the fronting edge of the leading peak

Table 3.7 and Table 3.8 depict observed response of experimental points for four different estrogenic hormones. Peak symmetry was calculated for all hormones. But resolution factor was calculated with respect to E1 which is the first peak in the chromatogram. The following response function was used for correlation of the resolution factor (RF) and peak symmetry (A_s) with independent parameters.

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{123}X_1X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 \quad (3.5)$$

Where Y is the predicted response for resolution factor (RF) and peak symmetry (A_s), b_0 is model constant, b_1, b_2, and b_3 are the linear coefficients, b_{12}, b_{13}, and b_{23} are the coefficients of interactions among the variables, and b_{11}, b_{22}, and b_{33} are the quadratic coefficients. The experimental results presented in Table 3.7 and Table 3.8 was used for determination of the response function coefficients in equation 3.5 by using Design Expert 7.0 statistic program for regression analysis.

Table 3.7 Observed peak symmetry of CCD for the hormones.

Run Number	Mobile Phase % ACN	Standard Solution %ACN	Flow Rate µL/min	PSE1	PSE2	PSE3	PS EE2
1	55.00	55.00	64.78	3.130	2.542	2.158	1.745
2	40.00	30.00	150.00	4.882	3.444	3.000	2.429
3	70.00	30.00	150.00	2.913	3.737	1.586	2.756
4	40.00	80.00	150.00	0.267	0.258	4.682	0.176
5	70.00	80.00	150.00	1.889	1.846	1.357	1.538
6	55.00	12.96	275.00	3.400	3.308	3.600	3.200
7	29.77	55.00	275.00	1.419	1.121	1.857	1.056
8	80.23	55.00	275.00	3.077	3.500	2.200	3.364
9	55.00	97.04	275.00	0.535	0.900	0.939	0.500
10	40.00	30.00	400.00	3.571	3.118	2.167	2.667
11	70.00	30.00	400.00	2.500	2.800	2.273	2.556
12	40.00	80.00	400.00	1.842	1.923	1.750	1.095
13	70.00	80.00	400.00	2.333	2.077	1.692	1.714
14	55.00	55.00	485.22	3.833	2.636	1.700	1.600
15-1	55.00	55.00	275.00	3.000	3.231	2.462	1.895
15-2	55.00	55.00	275.00	3.412	3.769	2.667	2.278
15-3	55.00	55.00	275.00	3.625	3.462	2.462	2.000
15-4	55.00	55.00	275.00	3.688	3.286	2.385	2.105
15-5	55.00	55.00	275.00	3.500	3.143	2.385	2.167

Table 3.8 Observed resolution factors of CCD for the hormones.

Run Number	Mobile Phase % ACN	Standard Solution %ACN	Flow Rate µL/min	RF E2	RF E3	RF EE2
1	55.00	55.00	64.78	0.191	1.077	0.407
2	40.00	30.00	150.00	0.688	2.806	0.412
3	70.00	30.00	150.00	0.066	0.154	0.167
4	40.00	80.00	150.00	0.245	0.744	0.101
5	70.00	80.00	150.00	0.000	0.250	0.134
6	55.00	12.96	275.00	0.208	0.714	0.234
7	29.77	55.00	275.00	1.000	2.585	0.283
8	80.23	55.00	275.00	0.000	0.156	0.102
9	55.00	97.04	275.00	0.066	0.341	0.133
10	40.00	30.00	400.00	0.441	1.698	0.328

Table 3.8 Observed resolution factors of CCD for the hormones (continued).

11	70.00	30.00	400.00	0.027	0.257	0.171
12	40.00	80.00	400.00	0.282	1.000	0.167
13	70.00	80.00	400.00	0.024	0.257	0.111
14	55.00	55.00	485.22	0.154	0.679	0.214
15-1	55.00	55.00	275.00	0.180	0.889	0.259
15-2	55.00	55.00	275.00	0.179	0.867	0.241
15-3	55.00	55.00	275.00	0.203	0.844	0.222
15-4	55.00	55.00	275.00	0.185	0.867	0.214
15-5	55.00	55.00	275.00	0.177	0.867	0.236

PS: Peak Symmetry

RF: Resolution Factor.

3.1.1 Optimization for Peak Symmetry

ANOVA analysis for the significance of coefficients of response equation for peak symmetry of hormones was conducted (Table 3.9). Values of "Prob > F" less than 0.0500 indicate model terms are significant. Values greater than 0.1000 indicate the model terms are not significant. Significance of quadratic coefficients indicate that response equation model is quadratic (as given in equation 3.5). Insignificant quadratic coefficients state that linear response equation can be used for diagnosis of model. The lack of fit test is desired to be insignificant which indicates that model fits and reliability of predicted responses. Reponses equation coefficients for different hormones were given in Table 3.10. R^2 >0.90 depicts a good agreement between predicted values and observed value. R^2 value was higher than 0.90 for E1, E2 and EE2 but less than 0.90 for E3. Adeq Precision" measures the signal to noise ratio. The ratio greater than 4 is desirable and states that model equation can be used to predict the reponse for any value of factors within the range of experimental design. Although R^2 value for E3 vas less than 0.90, Adeq Precison is larger than 4. Therefore, model coefficients can be used to predict response for E3.

Table 3.9 ANOVA analysis of peak symmetry for estrogenic hormones .

Source of variation		**E1**	**E2**	**E3**	**EE2**
Coded	**Actual**	**p-value Prob > F**	**p-value Prob > F**	**p-value Prob > F**	**p-value Prob > F**
M	Model	0.0012	0.0009	0.2876	0.0008
X_1	% ACN in mobile phase	0.2992	0.0039	0.1703	0.0008
X_2	% ACN in stationary phase	< 0.0001	< 0.0001	0.1791	< 0.0001
X_3	Flow rate	0.4040	0.5948	0.2342	0.4633
X_1X_2	Interatction between mobile phase and Stationary pahse	0.0039	0.1446	0.3498	0.0808
X_1X_3	% ACN in mobile phase vs Flow rate	0.8605	0.0983	0.0512	0.2181
X_2X_3	% ACN in stationary phase vs flow rate	0.0194	0.0202	0.2746	0.2658
X_1^2	Quadratic effect of mobile phase	0.0090	0.0101	0.6269	0.6847
X_2^2	Quadratic effect of stationary phase	0.0029	0.0037	0.9388	0.3098
X_3^2	Quadratic effect of flow rate	0.9025	0.0429	0.5149	0.1052
$X_1X_2X_3$	Three factor interaction	0.1526	0.4730	0.4274	0.7388
Lack of Fit		0.0840	0.1091	0.0004	0.0350

Table 3.10 Regression coefficients of peak symmetry response equation for estrogenic hormones.

Coefficient of response equation	**Coefficients for E1**	**Coefficients for E2**	**Coefficients for E3**	**Coefficients for EE2**
b_0	832.838	-102.270	+0.061779	318.706
b_1	+0.042668	+0.17090	+0.050065	-0.010742
b_2	-0.16219	-0.058248	+0.12648	-0.070689
b_3	-0.023429	3.23E+02	4.67E+02	5.50E+02
b_{12}	3.21E+02	1.19E+02	-1.97E+02	8.12E+01

Table 3.10 Regression coefficients of peak symmetry response equation for estrogenic hormones (continued).

b_{13}	2.82E+01	-1.56E+00	6.29E+00	-3.40E+00
b_{23}	4.47E+01	2.47E+01	-3.54E+01	8.70E+00
b_{11}	-1.87E+02	-1.56E+02	-4.49E+01	1.58E+01
b_{22}	-8.32E+01	-6.77E+01	-2.54E+00	-1.47E+01
b_{33}	9.94E-02	-1.61E+00	-8.72E-01	-9.88E-01
b_{123}	-5.41E-01	-2.19E-01	4.66E-01	-8.13E-02
R^2	0.9326	0.9362	0.6528	0.9384
Adequate precision	13.150	12.773	4.911	12.822

3.1.2 Effect of Independent Variables on Peak Symmetries of Estrogenic Hormones

The response equation developed for E1 was used to predict the any PS at different levels of independent variables. Then response surface plots were used to evaluate the effect these independent variables on the peak symmetry. The effect of flow rate and % ACN in standard solution (% ACN_s) on PS of E1 at constant ACN concentration in mobile phase (% ACN_m=40) was given in Figure 3.1. The increase in flow rate from 150 μL/min from 400 μL/min at the highest % ACN_s (90%) provided a slight improvement in PS value as PS=0.01 to 0.018, respectively. On the other hand, decreasing in %ACN_s from 90% to 15 % resulted in a very high PS value around PS= 4 to 5. This result indicates that low %ACN_s causes tailing in the chromatogram. PS=1 for E1 was obtained at %ACN_s= 80-0 and F=380 - 390 μL/min.

Variation of PS for E1 with % ACN_s and % ACN_m at constant flow rate, F= 300 μL/min, is depicted in Figure 3.2. Increasing percentage of ACN_m from 40% to 70% at high level of ACN_s (90%) provided a slight increase in PS from 0.99 to 1.49, respectively. It means that the effect of %ACN_m is not substantial on PS of E1. However, peak symmetry is destructed when %ACN_s is reduced to 15% at 40% ACN_m. This result indicates that there is a significant interaction between %ACN_s and %ACN_m.

The effect of flow rate and %ACN_m on PS of E1 at constant %ACN_s is given in Figure 3.3. The predicted PS value for the studied range of independent variables varied between 0 and 2.55. The highest destruction PS can be observed when ACN_m is increased from 40% to 70%. Flow has got a slight effect on PS destruction either. The most substantial effect of flow is observed when a percentage of ACN_m is 40%. PS can reach up to 2.55 which indicate a significant tailing in chromatogram. The conditions which results in PS around 1 can be determined as %ACN_m= 45-48 and flow is between 150-275 µL/min.

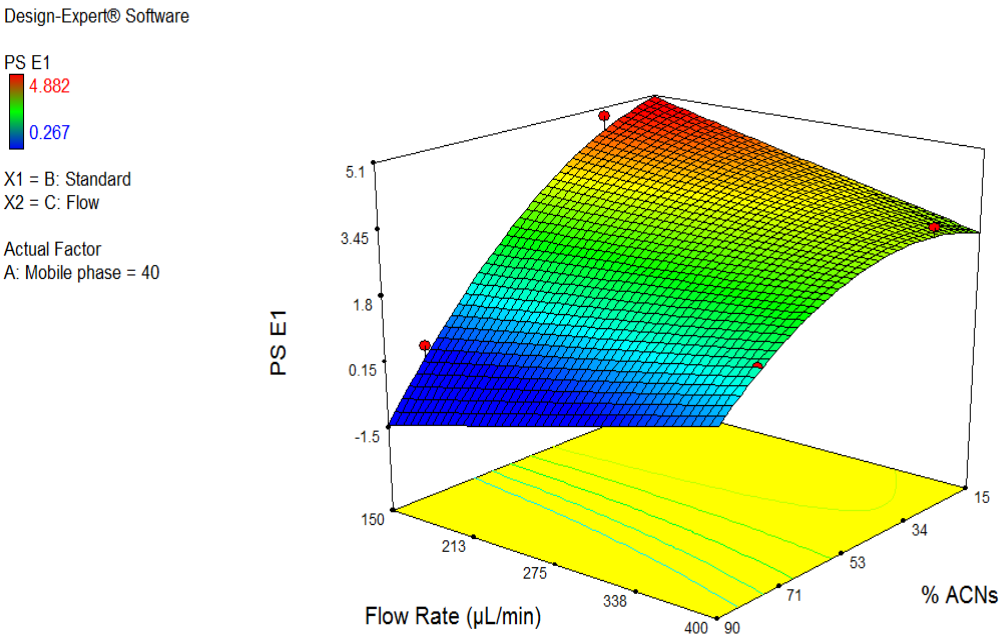


Figure 3.1 Variation of peak symmetry for E1 with flow and % ACN in standard at 40% ACN_m.

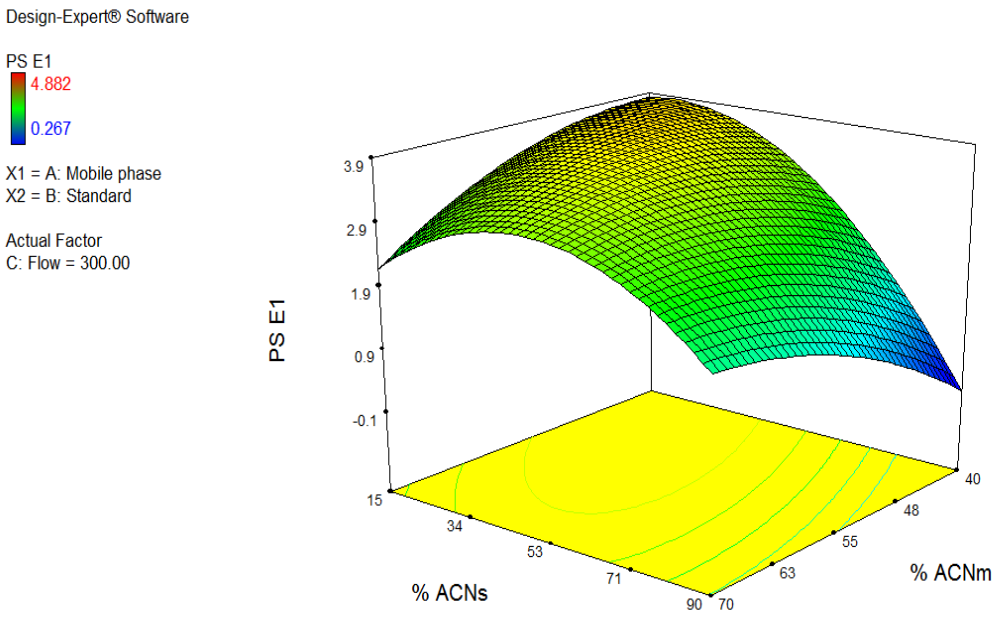


Figure 3.2 Variation of PS for E1 with % ACN standard solution and mobile phase at 300 µL/min.

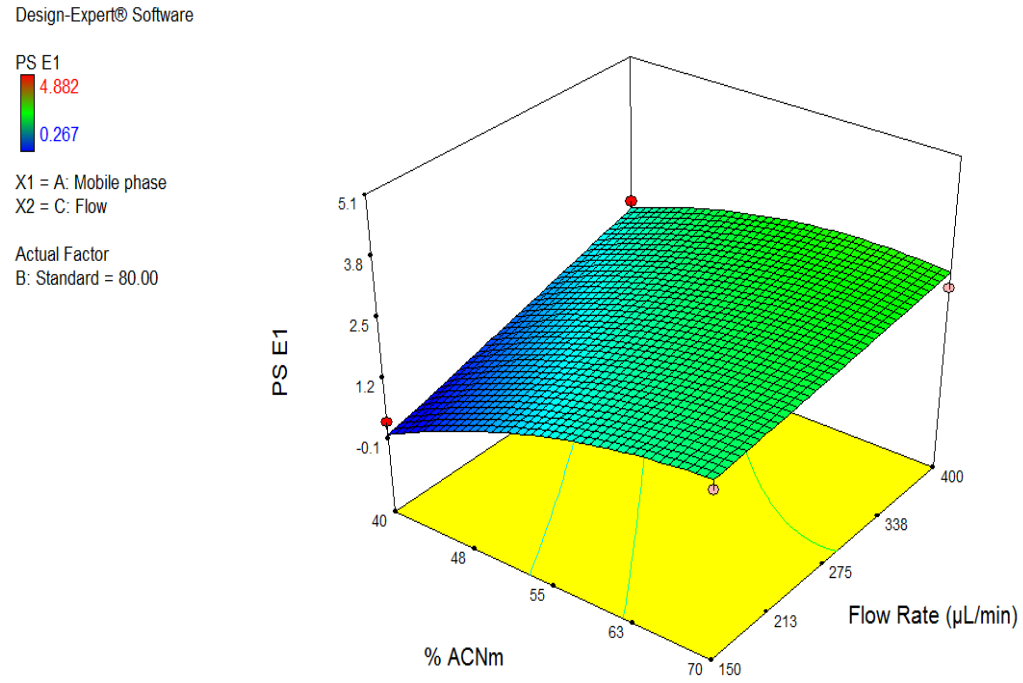


Figure 3.3 Variation of PS for E1 with % ACN_m and flow at % ACN_s= 80.

Effects of factors on PS of E2 were given through Figures 3.4 to 3.6. The first figure is about variation of PS for E2 with % ACN_s and %ACN_m at constant flow rate F= 300 µl/min. The most suitable peak symmetry value was observed when ACN_s is in the range of 90% to 70% for ACN_m between 40 - 70%. However, the

peak symmetry is substantially destructed when % ACN_s is less than 60% for any value of ACN_m. The effect of % ACN_m is not as significant as $\%ACN_s$ for E2.

Figure 3.5 indicates the effect of flow rate and $\%ACN_m$ on PS of E2 at constant $\%ACN_s$ (90%). The predicted PS values for the studied range of independent variables vary between 0 and 1.8. The highest destruction on PS can be observed when ACN_m is increased from 55% to 70%. Flow also has got a slight effect on PS structure. The most substantial effect of flow is observed when flow is higher than 325μL/min at ACN_m = 70%. PS can reach up to 1.425 which indicates a slight tailing in chromatogram. The conditions which results in PS around 1 can be determined as $\%ACN_m$ = 45-48 and flow is between 250-300 μL/min.

Similarly, effect of flow rate and % ACN_s on PS of E2 at constant ACN concentration in mobile phase (40%) was evaluated and 3D plot was given in Figure 3.6. At low ACN_s values between 60% - 30% for any flow rate, the peak symmetry was not acceptable. PS value range between PS = 2.0 - 3.4. However, increasing ACN in standard solution over 70% at 300μL/min >flow rates >250 μL/min resulted in a substantial improvement in the peak symmetry. The predicted PS values for these conditions were PS = 1.09 - 1.4. Therefore, the most suitable conditions to obtain PS values between 0.9 and 1.2 can be determined as $\%ACN_s$ > 80% and 260 μL/min > flow > 250 μL/min.

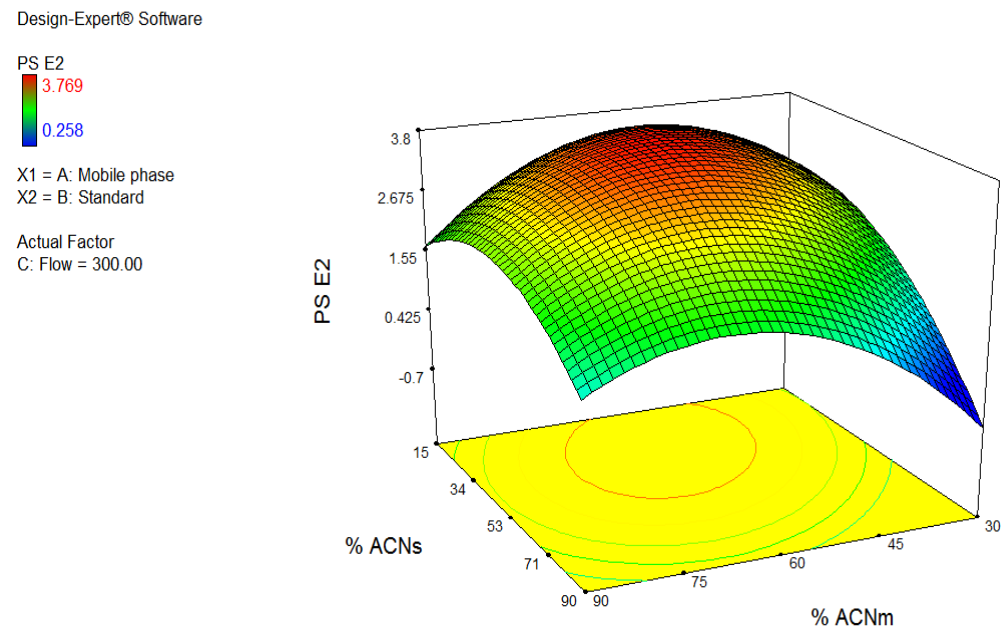


Figure 3.4 Variation of PS for E2 with % ACN standard solution and mobile phase at 300 µL/min flow.

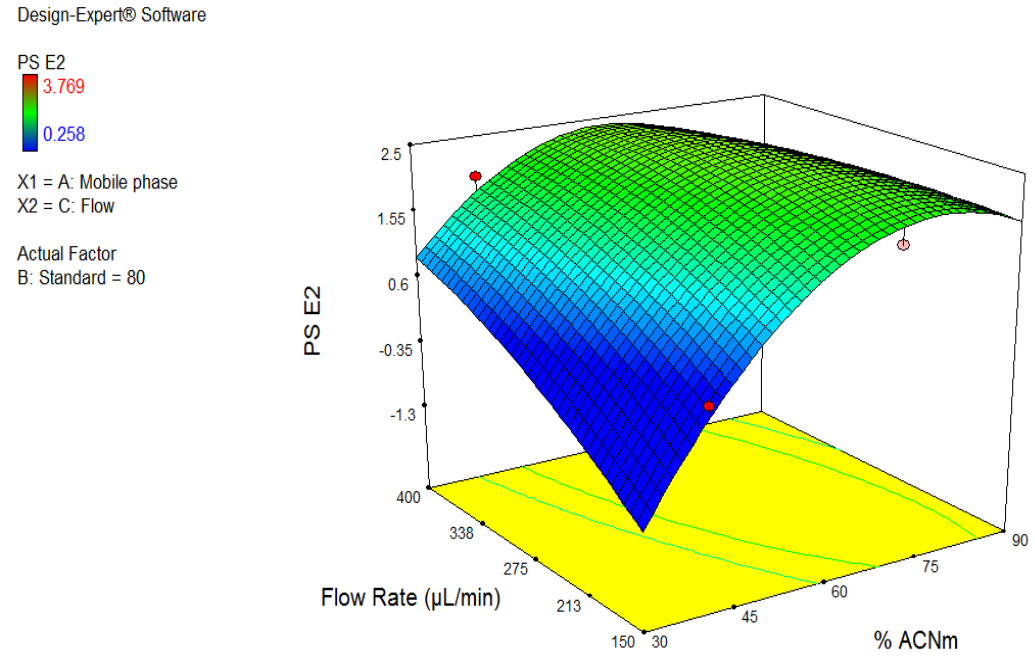


Figure 3.5 Variation of PS for E2 with % ACN_m and flow at % ACN_s= 80.

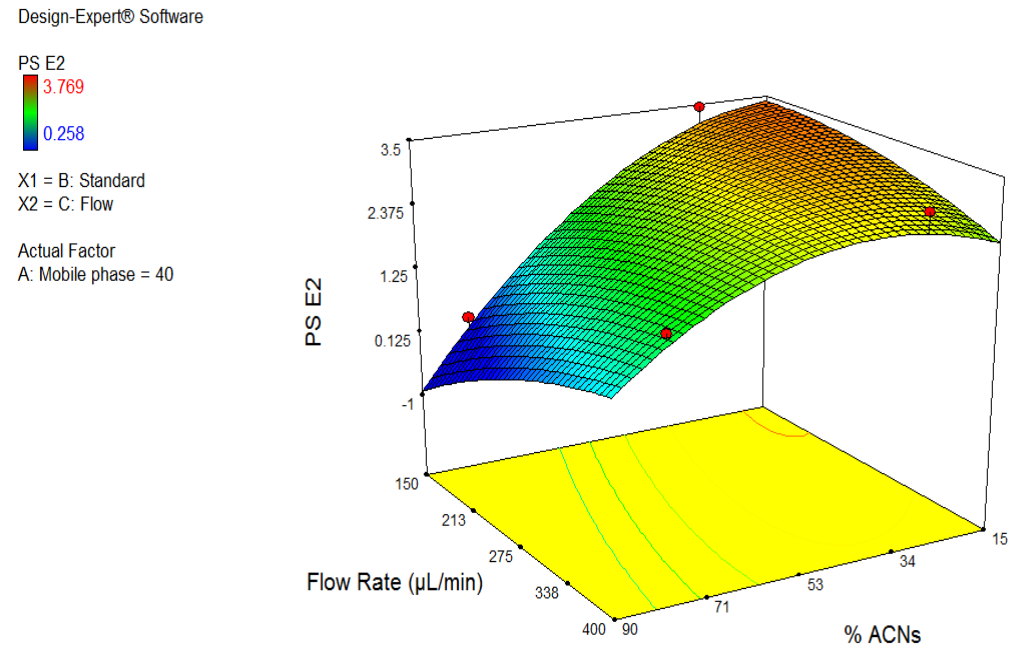


Figure 3.6 Variation of peak symmetry for E2 with flow and % ACN in standard at 40% ACN_m.

E3 is another estrogenic hormone encountered in wastewater and surface water. Peak symmetry of E3 was also considered throughout the experiments and statistical analysis of experimental results for linear response and quadratic response were conducted. Even though the highest correlation was observed for the quadratic response, observed and predicted values are not in good agreement (R^2 =0.65). It means, predicted values for any values of independent variables would not be representative of the real conditions. Therefore, predictions and evaluations of peak symmetry for E3 were not made.

EE2 is the only synthetic estrogenic hormone in the mixture of hormones used. In fact, it is the major concern in the wastewater. It does not form conjugates and hardly transformed to other forms. Statistical analysis for peak symmetry of EE2 indicated that model coefficients, $\%ACN_m$ and $\%ACN_s$ are significant factors that affect peak symmetry. Correlation coefficient was R^2=0.93 which proves a good agreement between observed and predicted values. Figure 3.7 depicts variation of PS of EE2 with flow and $\%ACN_s$ at ACN_m = 40%. The relationship between independent variables and response looks like linear, but there is slight curvation in the response.

% ACN_s has got a very significant effect on peak symmetry. Decrease in %ACN_s resulted in a significant destruction in peak symmetry. PS value was around 1 when ACN_s is 90% at flow 400 µL/min and then raised up to 3.0 when ACN_s= 15% at the same flow rate. Flow rate has not got a substantial effect on PS of EE2.

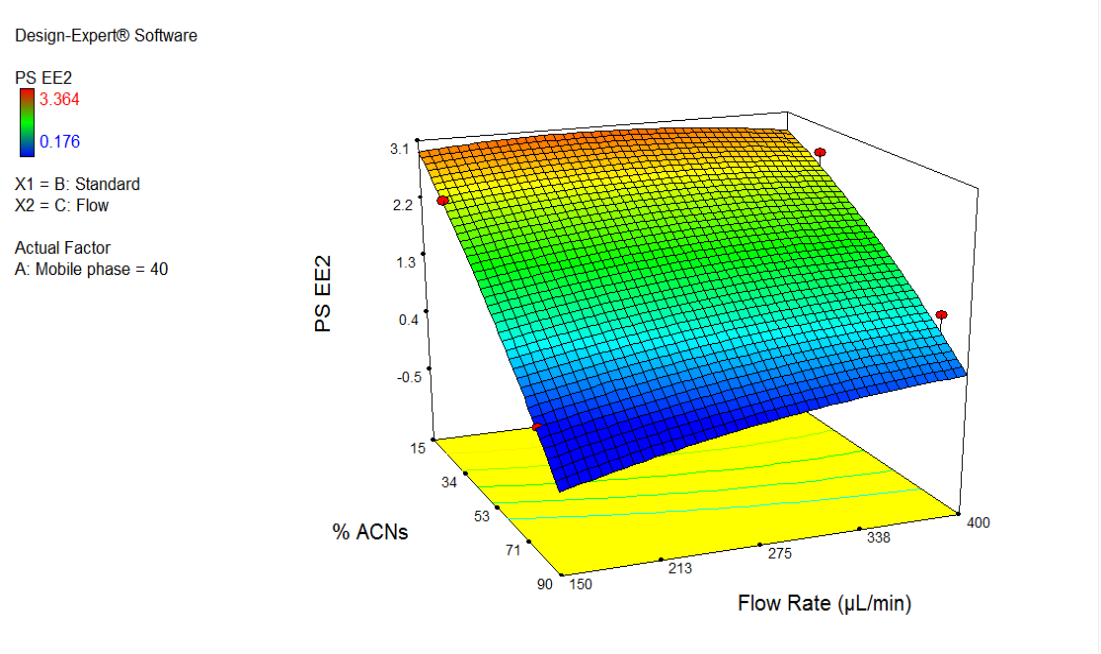


Figure 3.7 Variation of PS of EE2 with flow and %ACN_s at ACN_m = 40%.

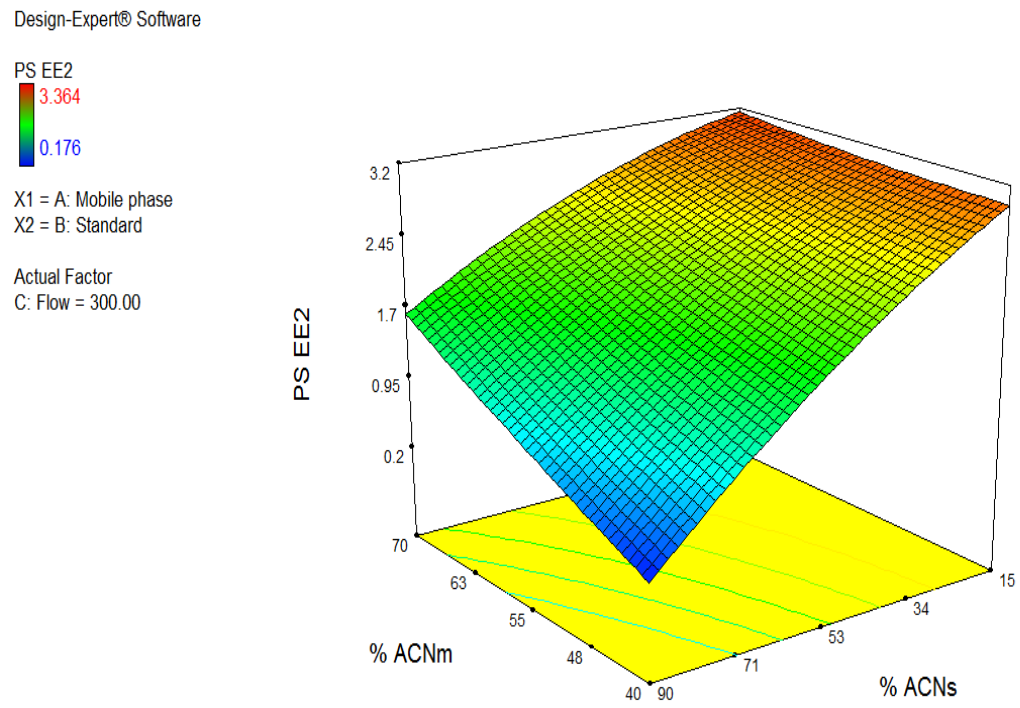


Figure 3.8 Variation of PS of EE2 with %ACN_m and %ACN_s at flow rate 300 µL/min.

Figure 3.8 shows effect of %ACN_m and %ACN_s on PS of EE2 at constant flow rate 300 µL/min. PS value increases from 0.3 to 1.7 linearly with the increase in mobile phase ACN concentration from 40% and 70% at 90% ACN_s. PS value varies between 0.77 to 1.7 for the suggested ACN_m concentrations. The most suitable ACN_m is 40% to 50% under this condition to obtain PS around 1. % ACN_s has got more significant effect on PS. The decrease in ACN_s from 90% to 15% for 40% ACN_m resulted in a significant shift in PS from 0.2 to 3.0 which indicate a considerable tailing in chromatogram for these extreme conditions. For the best PS value of EE2 chromatogram, % ACN_m should be around 70%.

3.1.3 Effect of Independent Variables on Resolution Factors

Resolution factor was the second response in CCD. In other words, resolution factor was also calculated for the same experimental conditions as conducted for peak symmetry. Table 3.11 depicts the variance analysis of peak symmetry for E2, E3 and EE2. Resolution factor of E2 was calculated with regard to E1. Therefore, no significance analysis was carried out for E1. As seen from the table, %ACN_s, % ACN_m and flow are significant factors for RF of E2 and E3. However, flow is not significant for Rf of EE2. Since retention time of EE2 is quite longer than that of other hormones. All two level interactions for E2 and E3 are significant. Only, interaction between % ACN_m and % ACN_s is significant for EE2. In summary, the resolution factor should be optimized for all factors in the case of E2 and E3, but it is only necessary to conduct optimization of RF with regard to % ACN_s and %ACN_m in the case of EE2.

The following response equation 3.6 was developed and coefficients were determined by regression analysis (Table 3.12). Predicted responses for any value of studied range of factors were calculated by using these coefficients. 3D plots were used to evaluate the effect of the factors on resolution factors.

$$Y= b_0 + b_1X_1 + b_2X_2 + b_3X_3+b_{12}X_1X_2 + b_{13} X_1X_3+b_{23}X_2X_3+b_{123}X_1X_2X_3+ b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 \quad (3.6)$$

Table 3.11 ANOVA analysis of resolution factor for estrogenic hormones.

Source of variation		**E2**	**E3**	**EE2**
Coded	**Actual**	**p-value Prob > F**	**p-value Prob > F**	**p-value Prob > F**
M	Model	< 0.0001	< 0.0001	0.0105
X_1	% ACN in mobile phase	*< 0.0001*	*< 0.0001*	*0.0036*
X_2	% ACN in stationary phase	*0.0003*	*0.0003*	*0.0034*
X_3	Flow rate	*0.0345*	*0.0345*	*0.0777*
X_1X_2	Interatction between mobile phase and Stationary pahse	*0.0001*	*0.0001*	*0.0244*
X_1X_3	% ACN in mobile phase vs Flowrate	*0.0533*	*0.0533*	*0.9994*
X_2X_3	% ACN in stationary phase vs flowrate	*0.0174*	*0.0174*	*0.4016*
X_1^2	Quadratic effect of mobile phase	*0.0030*	*0.0030*	*0.1425*
X_2^2	Quadratic effect of stationary phase	*0.0141*	*0.0141*	*0.0998*
X_3^2	Quadratic effect of flowrate	*0.9401*	*0.9401*	*0.1548*
$X_1X_2X_3$	Three factor interaction	*0.0088*	*0.0088*	*0.2316*
Lack of Fit		*0.3656*	*< 0.0001*	*0.0122*

Table 3.12 Regression coefficients of resolution factor response equation for estrogenic hormones.

Coefficient of response equation	**Coefficients for E2**	**Coefficients for E3**	**Coefficients for EE2**
b_0	+3.80738	+13.67961	1.15165
b_1	-0.086864	-0.25848	-7.23E+02
b_2	-0.020127	-0.11250	-0.013287
b_3	-2.86E+02	-0.018813	-2.63E+02
b_{12}	3.39E+01	2.02E+02	2.56E+01
b_{23}	4.53E+00	2.78E+01	2.60E+00
b_{23}	4.62E+00	2.65E+01	3.08E+00
b_{11}	4.45E+01	7.60E+01	-9.47E+00
b_{22}	-4.55E+00	-2.03E+01	-3.90E+00

Table 3.12 Regression coefficients of resolution factor response equation for estrogenic hormones (continued)

b_{33}	-1.01E-01	-2.02E-02	1.32E-01
b_{123}	-5.87E-02	-3.89E-01	-4.73E-02
R^2	0.9526	0.9819	0.8772
Adequate precision	14.231	23.622	9.570

Figure 3.9 depicts variation of resolution factor of E2 with respect to E1 at different %ACN_s and % ACN_m concentrations (flow = 300 μL/min). The higher the RF, better the resolution. Therefore , figure were plotted to obtain the highest RF values. As seen from figure, the highest RF value (0.64) is obtained at the lowest concentration of ACN in mobile phase and in standard solution. The effects of different flow rate and %ACN_m on RF of E2 at %ACN_s = 25 is given in Figure 3.10. RF increases from 0.01 to 0.8 at the lowest flow rate of 150 μL/min when % ACN_m was decreased from 70% to 40%. On the other hand, increasing flow rate from 150 to 400 μL/min at 40% ACN_m does not provide a substantial improvement in RF value of E2. There is an only increase from 0.4 to 0.8 in RF for the mentioned conditions. Therefore, it can be concluded that % ACN_m is more significant factor than flow in the resolution of E1 and E2 peaks. Finally, variation of RF with flow and %ACN_s at 40% ACN_m was evaluated and results were given in Figure 3.11. The highest RF value was observed as 0.82 at flow= 150 μL/min and % ACN_s= 15%. The effect of %ACN_s is more subtantial at flow rate= 150 μL/min compared to flow= 400 μL/min. The RF rises from 0.2 to 0.8 for the former case. In summary, the results indicate that the maximum RF value around 0.8 for E2 can be obtained at flow rate= 150 μL/min, % ACN_m= 40 and % ACN_s= 15.

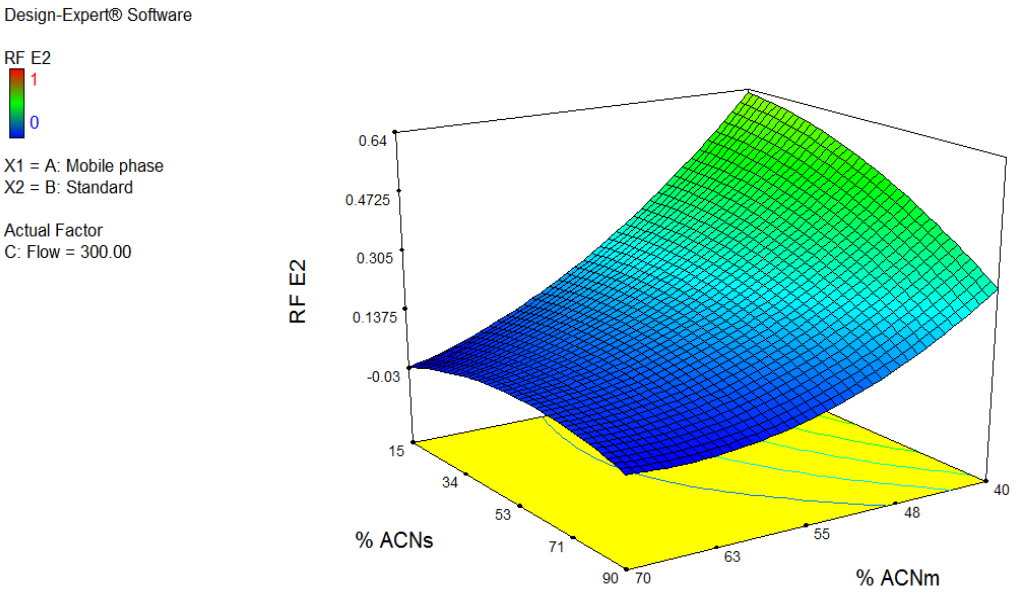


Figure 3.9 Variation of RF of E2 with $\%ACN_m$ and $\%ACN_s$ at flow rate 300 µL/min.

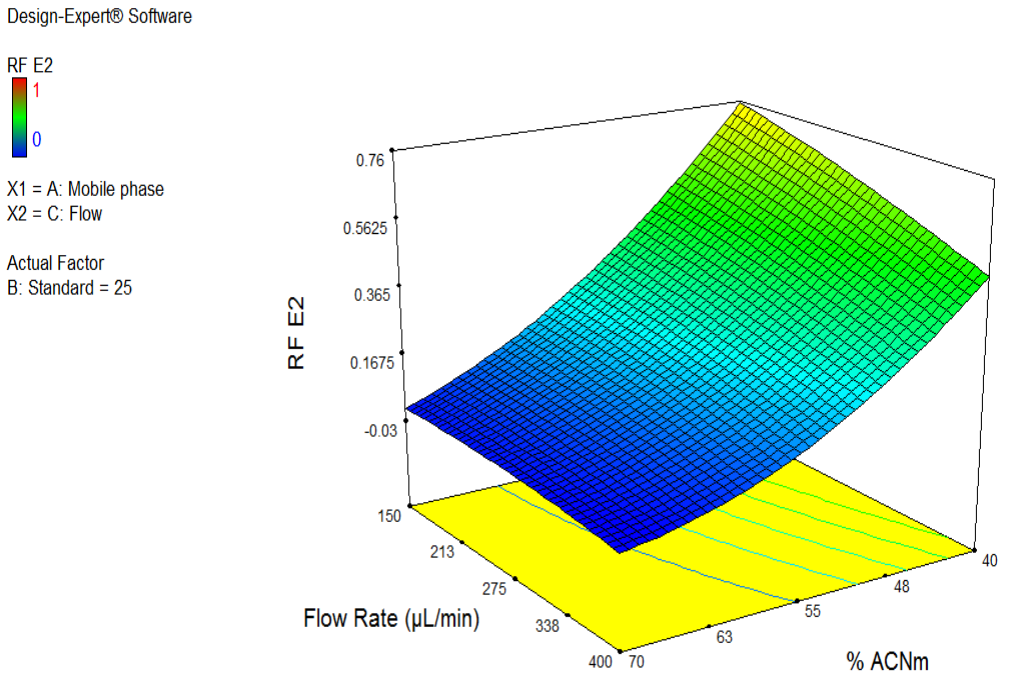


Figure 3.10 Variation of RF of E2 with $\%ACN_m$ and flow rate at $\%ACN_s$ = 25.

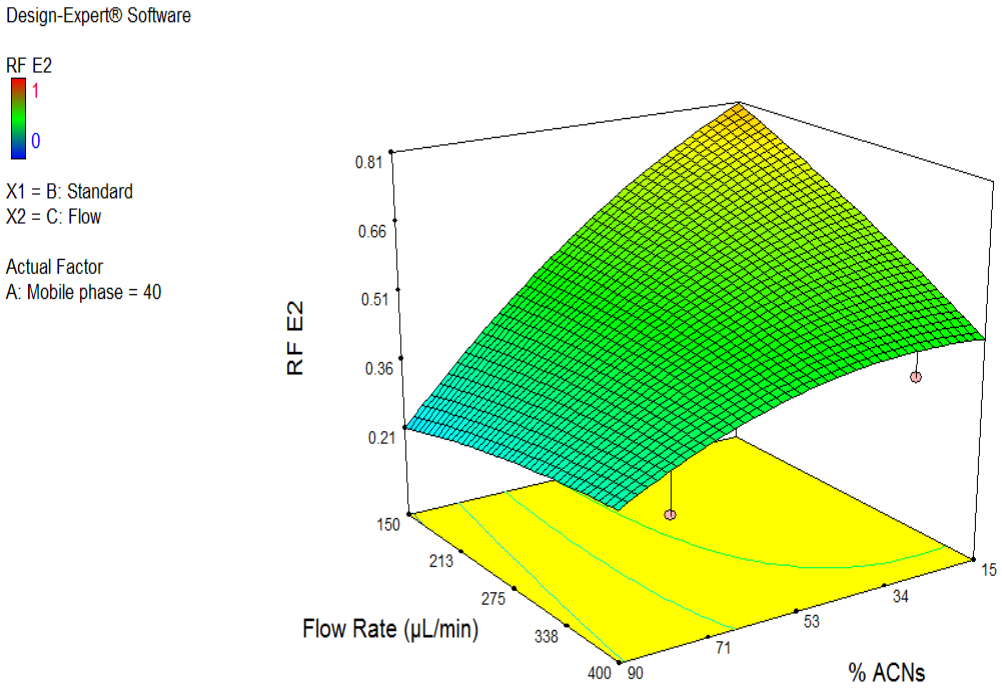


Figure 3.11 Variation of RF of E2 with flow and %ACN_s at 40% ACN_m.

E3 is eluted from the coloum after E2. Therefore resolution factor of E3 was calculted with regard to E2. Figure 3.12 shows of resolution factor of E2 at different %ACN_s and % ACN_m concentrations (flow = 300 µL/min). The highest resolution factor is obtained as RF=2.3 when ACN_m= 40% and ACN_s= 15 %. However, almost no resolution can be obtained for 70% ACN_m. Similarly, resolution was too low at 90% ACN_s any value.

The effects of different flow rates and %ACN_m on RF of E3 at %ACN_s = 25 is given in Figure 3.13. RF increases from 0.00 to 3 at the lowest flow rate of 150 µL/min when % ACN_m was decreased from 70% to 40%. On the other hand, increasing flow rate from 150 to 400 µL/min at 40% ACN_m does not provide a substantial improvement in RF value of E3. The increse in RF value was only from 2.1 to 3.0. Therefore, it can be concluded that % ACN_m is more signficant factor than flow in the resolution of E2 and E3 peaks.

Finally, the highest value of RF (3.2) of E3 was observed at conditions when flow rate and ACN_s are minimum (ACN_m= 40%) as given in Figure 3.14. The effect of %ACN_s is more subtantial at flow rate= 150 µL/min compared to flow= 400 µL/min.

In summary the results indicate that maximum RF value around 3 for E3 can be obtained at flow rate= 150 µL/min, % ACN_m= 40 and % ACN_s= 15.

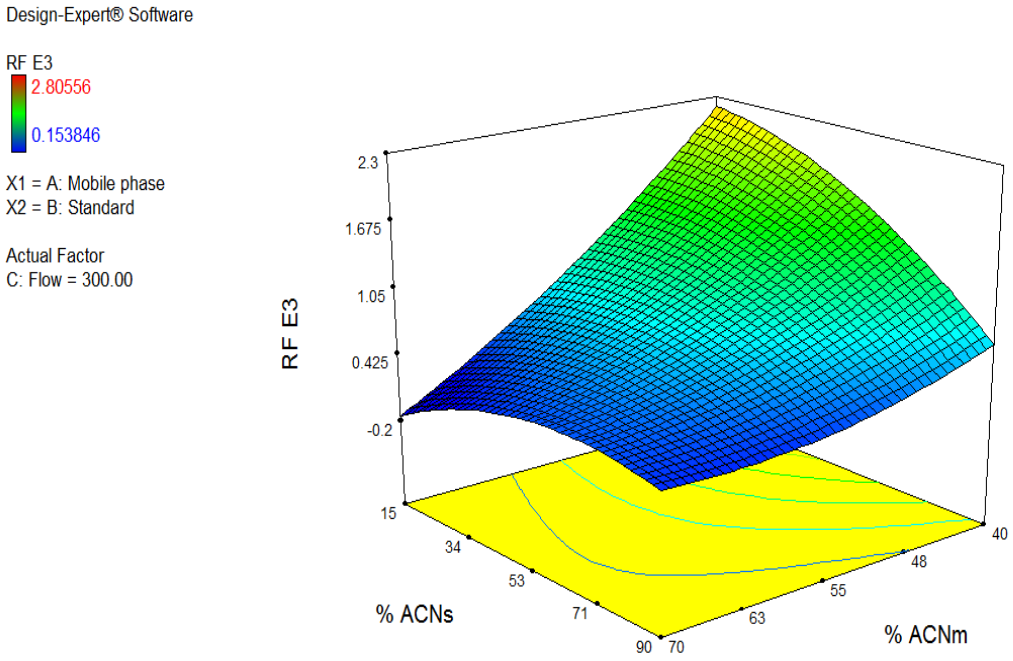


Figure 3.12 Variation of RF of E3 with % ACN_m and %ACN_s at flow rate 300 µL/min.

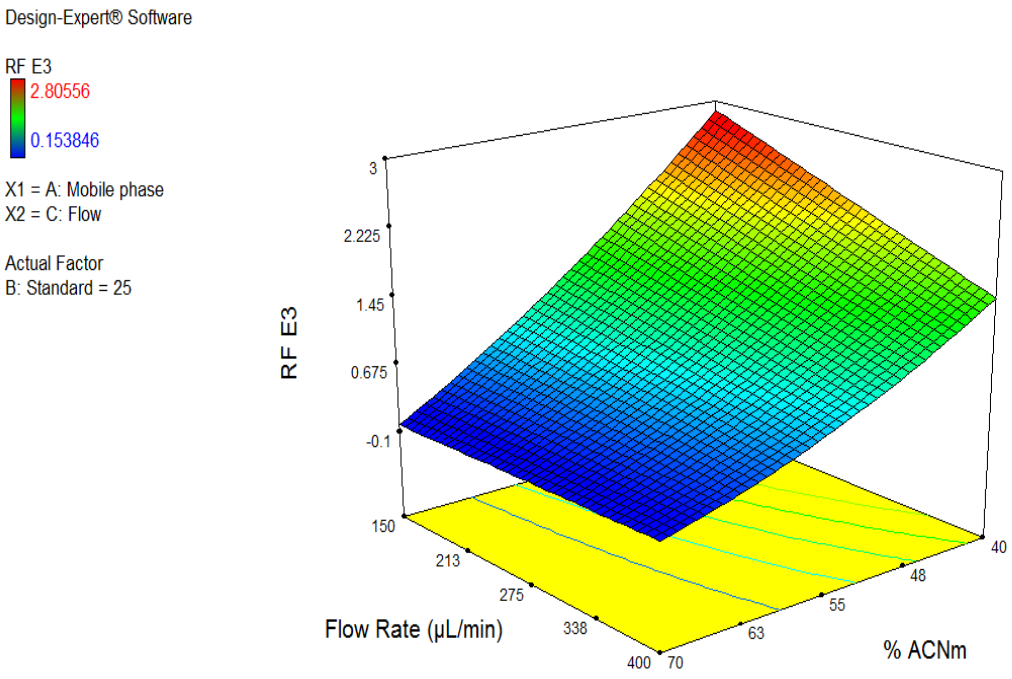


Figure 3.13 Variation of RF of E3 with % ACN_m and flow rate at 25 %ACN_s.

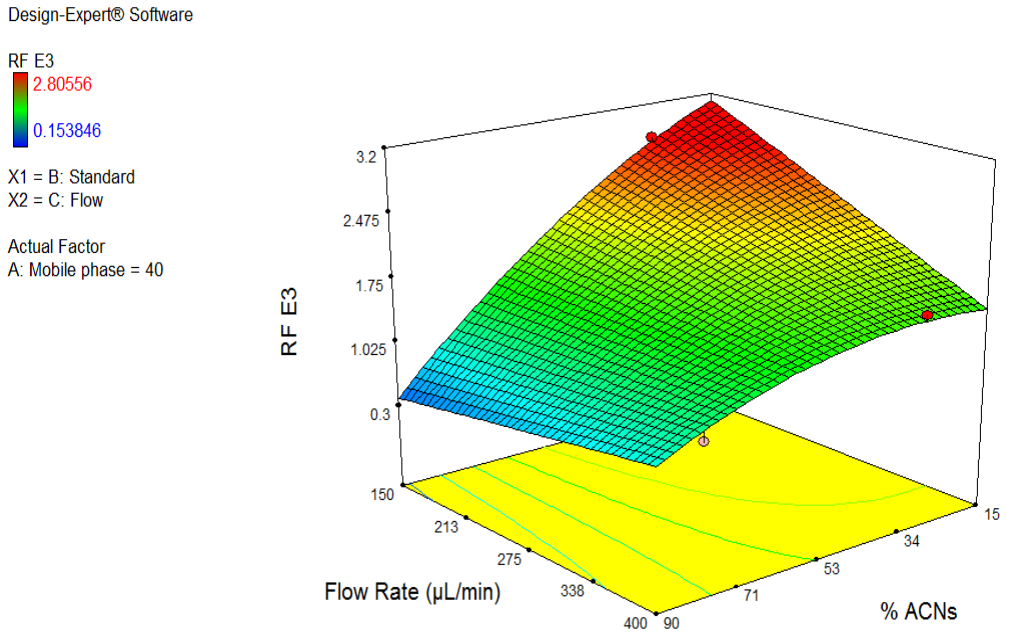


Figure 3.14 Variation of RF of E3 with %ACN_s and flow rate at 40 % ACN_m.

Under the different %ACN_s and % ACN_m conditions, variation of RF of EE2 was shown in figure 3.15. The flow rate was kept constat at 300 µL/min in this figure. %ACN_s can be said insignificant factor for RF of E3 at 70 % ACN_m. However,

when ACN_m is decreased from 70% to 40%, ACN_s becomes a signficant factor. Similarly, no significant variation in RF is observed for different ACN_s at 70% ACN_m. However, the combined effect of these two factor when they are at low level increases the response from 0.09 to 0.36.

Figure 3.16 depicts variation of resolution factor of EE2 with respect to E3 at different % ACN_m and flow rate when %ACN_s = 25. As seen from figure, the highest RF value (0.45) is obtained at the lowest concentration of ACN in mobile phase and lowest flow rate. Finally, variation of RF with flow and %ACN_s at 40% ACN_m was evaluated and results are given in Figure 3.17. The highest RF value was observed as 0.48 at flow = 150 µL/min and % ACN_s= 15%. The effect of %ACN_s is more subtantial at flow rate= 150 µL/min compared to flow= 400 µL/min. In summary the results indicate that the maximum RF value around 0.4 for EE2 can be obtained at flow rate= 150 µL/min, % ACN_m= 40 and % ACN_s= 15.

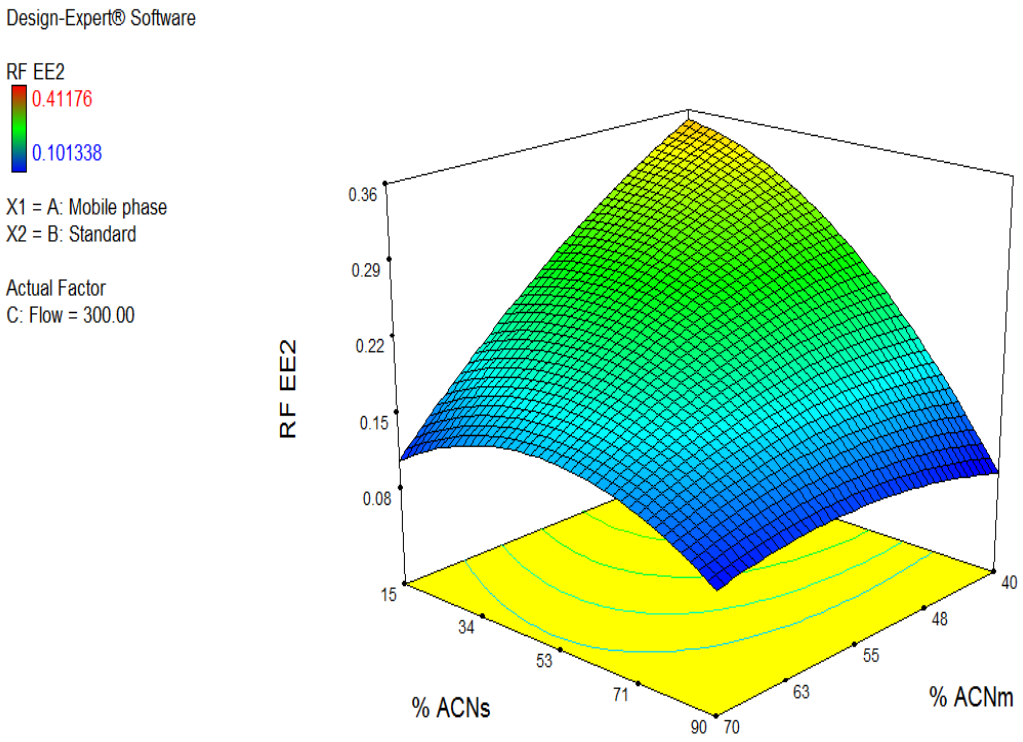


Figure 3.15 Variation of RF of EE2 with % ACN_m and %ACN_s at flow rate 300 µL/min.

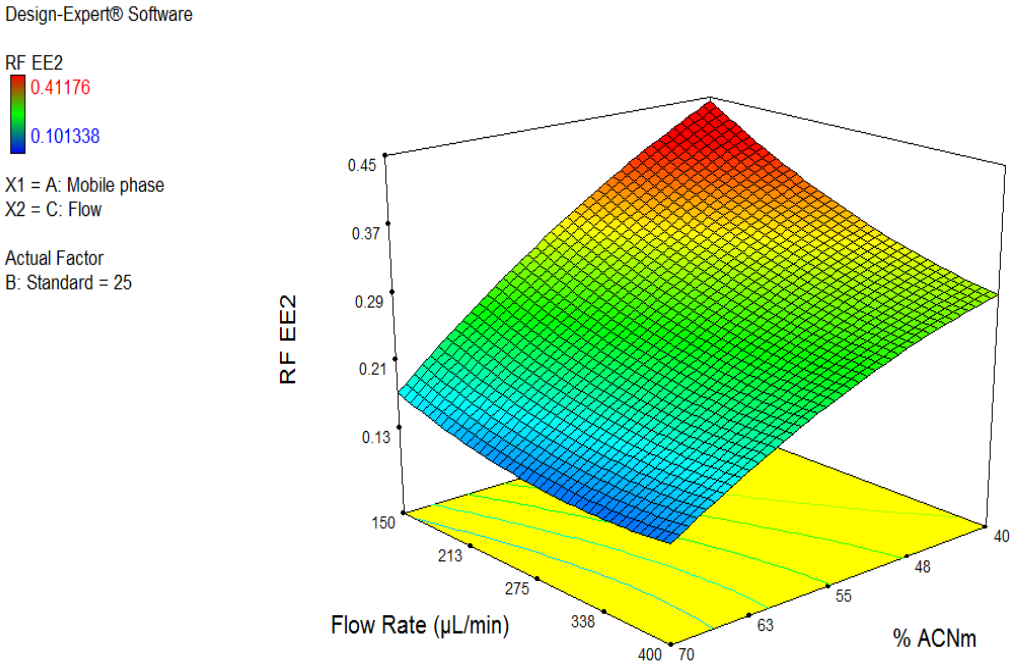


Figure 3.16 Variation of RF of EE2 with % ACN_m and flow rate at 25 %ACN_s.

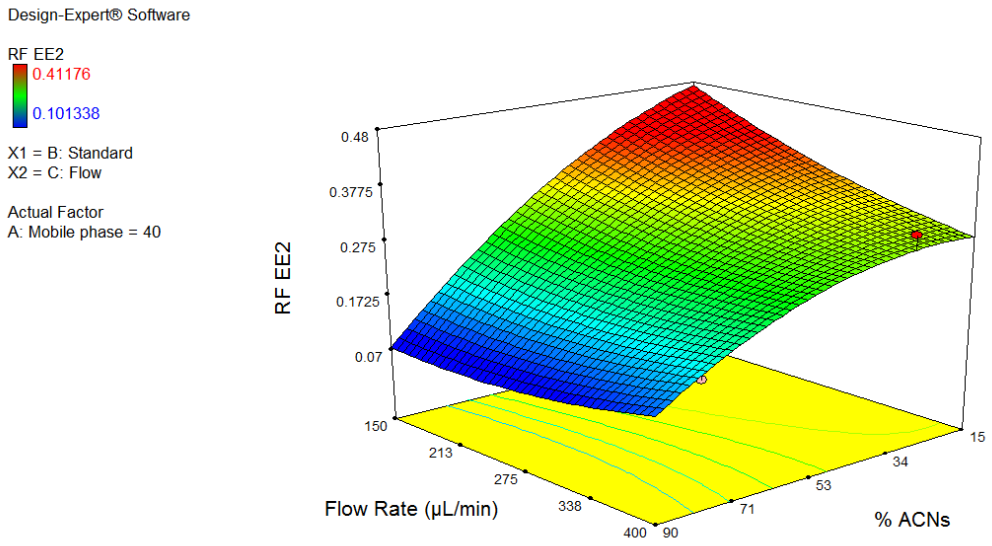


Figure 3.17 Variation of RF of EE2 with %ACN_s and flow rate at 40 % ACN_m.

Table 3.13 indicates the observed and predicted values for the experimental points which are different than the design points. This study was conducted to ensure that response equations can be used to predict the response. The observed and predicted values are very close to each other for most of the cases. This result indicates that response equations developed for RF can be used to predict RF values for any values of independent variables.

Table 3.13 Model verification at conditions different than design points.

Experimental Points	Mobile Phase % Acn	Standard Solution % ACN	Flow Rate µL/min	RF E2 (Predicted / Observed)	RF E3 (Predicted / Observed)	RF EE2 (Predicted / Observed)
2-1	45	25	250	0.5025 / 0.3506	1.8993 / 1.3571	0.3425 / 0.3750
6-1	50	20	200	0.4011 / 0.3150	1.6411 / 1.0000	0.3430 / 0.3442
6-2	55	15	400	0.1146 / 0.2187	0.5684 / 0.8214	0.2292 / 0.2857
10-1	40	25	300	0.615 / 0.6610	2.1365 / 1.7407	0.3406 / 0.5348
15-1	55	55	450	0.1267 / 0.1515	0.7171 / 0.6667	0.2391 / 0.2188

In summary, the common operation conditions to get the best chromatogram in terms of peak symmetry were determined as % ACN_m= 40, %ACN_s= 25 and flow= 300 µL/min. The peak symmetry can be obtained around PS=1 under this conditions The conditions for resolution factor were as flow rate= 150 µL/min, % ACN_m= 40 and % ACN_s= 15. However, peak symmetry was selected as more imported for the chromotograms. In addition, resolution factor obtained as acceptable level around 0.6 to 1.7 depending on the hormone type for the former conditions. Figure 3.18 shows the chromatogram of 4 hormones obtained under this operation conditions.

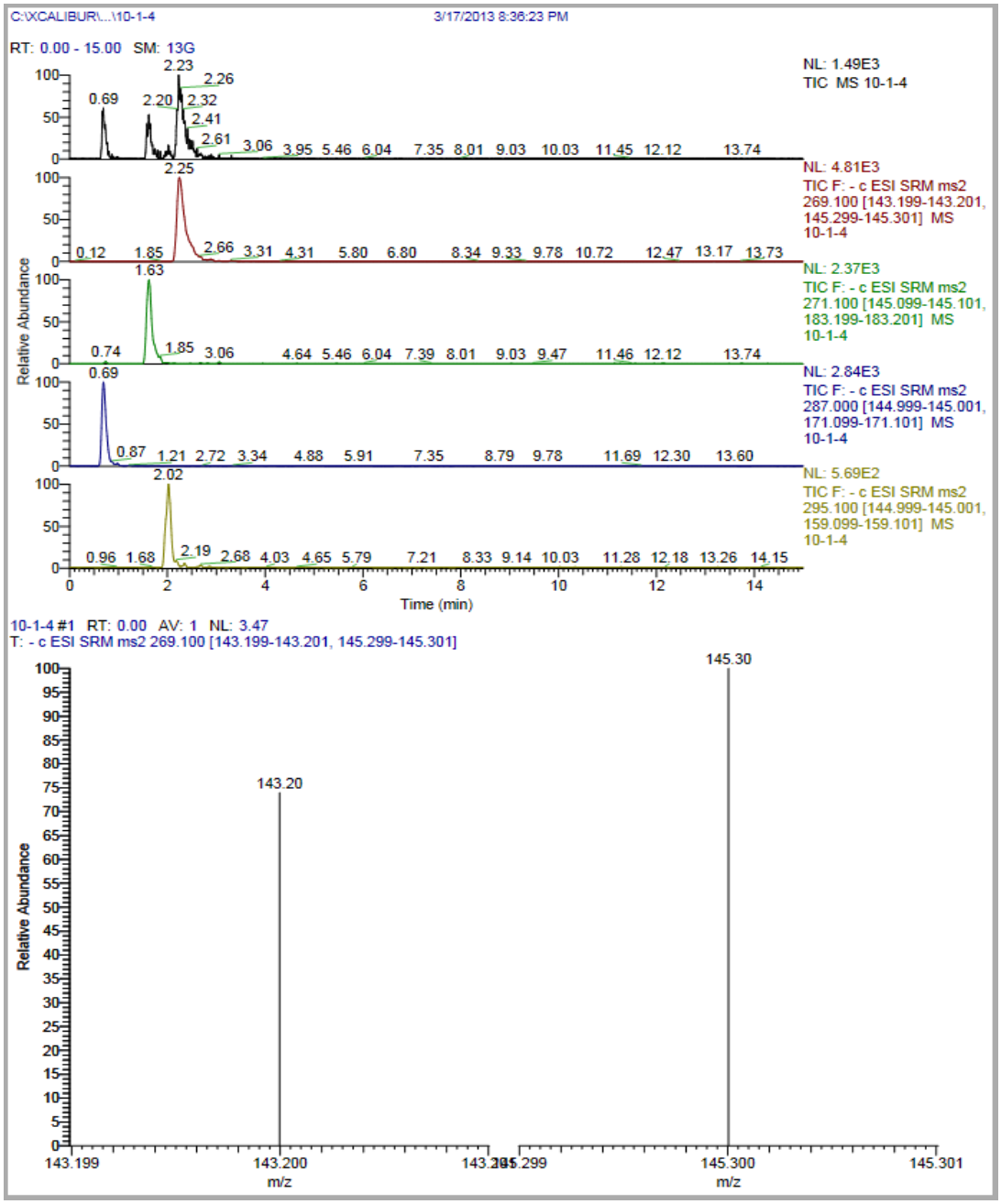


Figure 3.18 Chromatogram of four hormones obtained under this operation conditions.

3.2 The Effect of NH_4OH Concentration on Integration Area of Estrogenic Hormones

Some studies reported that addition of NH_4OH helps the ionization of hormones and increasing in integration area of hormones. In order to investigate the effect of NH_4OH concentration, the experiments were conducted at different NH_4OH (200mM in water) flow ratio between 3% and 17% in mobile phase and concentration of

standard hormones solution to inject LC-MS/MS was fixed at 50 µg/L. Control experiments without NH_4OH were conducted in parallel to the experiments with NH_4OH. NH_4OH was prepared in stock as 200 mM concentration and given to the colon from different line. % ACN in mobile phase was 40% and the flow rate was 300 µL/min as determined in the previous section. No gradient was applied. The operation conditions for LC-MS/MS were summarized in Tables between 3.14 and 3.17.

Single factor experimental design method was used in order to evaluate if there is significant difference on integration area of the hormones for different concentrations of NH_4OH. The experiments were repeated 10 times. Variance analysis was conducted to determine if the concentration is a significant factor. Then Least Significant Difference test (LSD) was applied to the data to select the NH_4OH concentration which results in significantly higher integration area.

Table 3.14 The auto sampler conditions for determination of $\%NH_4OH$ effect on integration area.

Injection Volume (µL)	25 µL
Needle Height from Bottom (mm)	0.1
Syringe Speed (µL/s)	5
Flush Volume (µL)	400
Flush / Wash Source	Wash Bottle
Wash Volume (µL)	3000
Flush Speed (µL/s)	100
Post-Injection Valve Switch Time (min)	0
Injection Mode	Full Loop
Tray Temperature (^oC)	25
Column Oven Temperature (^oC)	25

Table 3.15 Selected reaction mode conditions for determination of $\%NH_4OH$ effect on integration area.

MS Acquire Time (min)	15
Collision Gas Pressure (m Torr)*	1.5
Cycle Time (S)	0.5

(*) 1 mTorr = 0.000001315789473684 atm = 0.000001333223684211 bar

Table 3.16 SRM conditions for determination of %NH_4OH effect on integration area.

	Parent Mass	Product Mass	SRM Collision Energy	Retention Time	Time Window	Tube Lens	Polarity	Trigger	Name
1	269.1	143.2	51	7.50	15.00	85	-	0	E1
2	269.1	145.3	38	7.50	15.00	85	-	0	E1
3	271.1	145.1	43	7.50	15.00	85	-	0	E2
4	271.1	183.2	41	7.50	15.00	85	-	0	E2
5	287.0	145.0	37	7.50	15.00	85	-	0	E3
6	287.0	171.1	38	7.50	15.00	85	-	0	E3
7	295.1	145.0	45	7.50	15.00	85	-	0	EE2
8	295.1	159.1	37	7.50	15.00	85	-	0	EE2

Table 3.17 The interface conditions for determination of %NH_4OH effect on integration area.

Capillary Temperature (^oC)	280
Vaporizer Temperature (^oC)	120
Sheath Gas Pressure (Arb)	25
Aux Gas Pressure (Arb*)	20
Ion Sweep Gas Pressure (Arb)	2.0
Spray Voltage (V) (positive/negative polarity)	2800/3500

$^{(*)}$Arb (arbitrary)

3.2.1 Effect of % NH_4OH on Integration Area of E1

Tables 3.18 depict the variance analysis of different NH_4OH concentrations on integration area (A) of E1. The statistical analysis indicated that concentration of NH_4OH significantly affect the response area. The expectation with the addition of NH_4OH was increase in the response area with the increase in NH_4OH concentration. Figure 3.19 shows that mean integration area of E1 for 10 replicates at different NH_4OH concentrations. It was A=369647 when no NH_4OH was added into mobile phase (See appendix Table 1). However mean area increased to A=840446 for 3% NH_4OH. A slight increase to A=851879 was observed at 5% NH_4OH. On the other hand, further increase in NH_4OH resulted in decreasing in mean integration area. The resulting mean area varied between A=756640 and A=572232 for NH_4OH concentrations 7% to 17%, respectively. These results indicate that the highest peak areas can be obtained at NH_4OH = 3%-5%. LSD test indicated that there is no

significant difference between 3% and 5% in terms of the integrating area (See appendix Table 2). Therefore, the lowest concentration of NH_4OH to get the highest integration area of E1 can be determined as 3%.

Table 3.18 ANOVA for integration area of E1 at different NH_4OH concentrations.

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.69E+12	6	2.81086E+11	22.97206454	7.07E-14	2.25405301
Within Groups	7.34E+11	60	12235992113			
Total	2.42E+12	66				

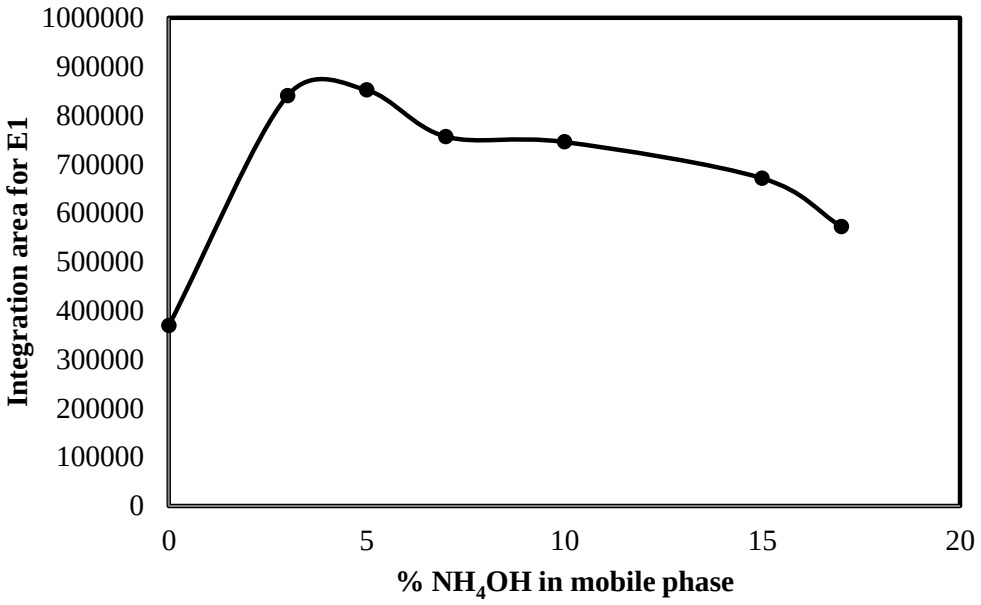


Figure 3.19 Variation of integration area of E1 with % NH_4OH concentration in mobile phase.

3.2.2 Effect of % NH_4OH on Integration Area of E2

The effect of NH_4OH concentration on ionization of E2 was almost the same as E1. The statistical analysis indicated that concentration of NH_4OH significantly affected the response area (Table 3.19). Variation of E2 integration area with NH_4OH is depicted in Figure 3.20. Mean integration area was 165799 when no NH_4OH was added into mobile phase (See appendix Table 3). A substantial increase

to A=388481 and A=408593 when NH_4OH was increased to 3% and 5%, respectively. Higher concentrations of NH_4OH adversely affected the area which varied between A=324443 and A=288114 for NH_4OH concentrations 7% to 17%, respectively. As a result of this study, the maximum area were obtained at NH_4OH= 3%-5%. LSD test indicated that there is no significant difference between 3% and 5% in terms of the integrating area (See appendix Table 4). Therefore, the lowest concentration of NH_4OH to get the highest integration area of E2 can be determined as 3%.

Table 3.19 ANOVA analysis of different NH_4OH concentration in mobile phase on integration area of E2.

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	348233254464.299	6	58038875744.050	49.711	0.000	2.275
Within Groups	61878906924.108	53	1167526545.738			
Total	410112161388.407	59				

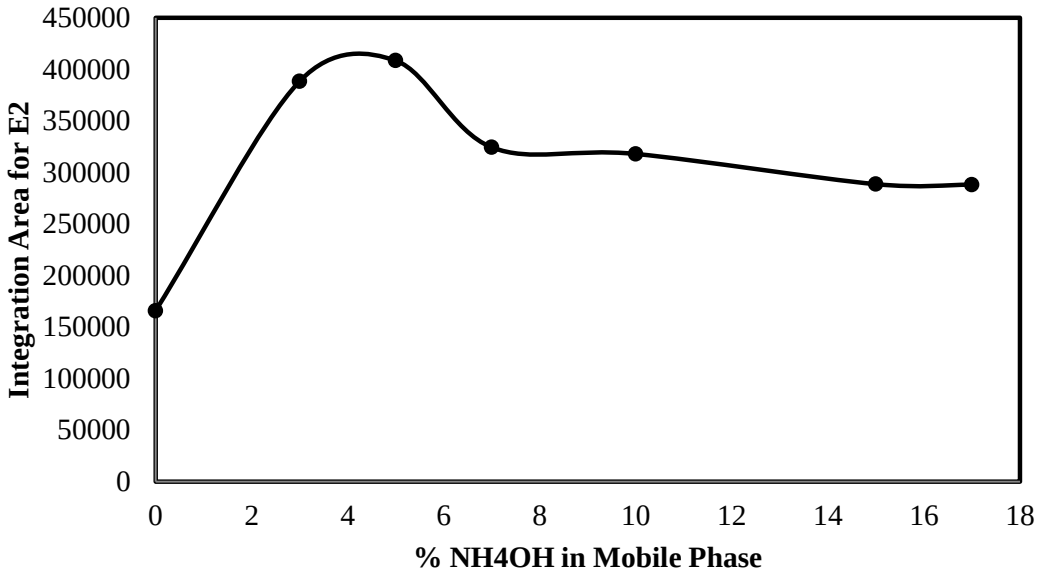


Figure 3.20 Variation of integration area of E2 with % NH_4OH concentration in mobile phase.

3.2.3 Effect of % NH_4OH on Integration Area of E3

The maximum integration area of E3 was obtained at the same NH_4OH concentrations as it was obtained for other hormones. NH_4OH concentration was significant for integration area of E3 (Table 3.20). Similar to the other hormones, the maximum area was achieved at 3% and 5% concentrations (See appendix Table 5) and response decreased for higher concentrations of NH_4OH. LSD test also proved that the integration areas for 3% or 5% were significantly different than no NH_4OH added or NH_4OH>5% conditions. The comparison between 3% and 5% resulted in that both concentrations give the same area (See appendix Table 6). For the sake of process simplicity and economy, 3% can be selected as optimal concentration for E3, too.

Table 3.20 ANOVA for integration area of E3 at different NH_4OH concentrations .

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.88E+11	6	31312780357	18.3729824	6.67E-12	2.25678
Within Groups	1.01E+11	59	1704284025			
Total	2.88E+11	65				

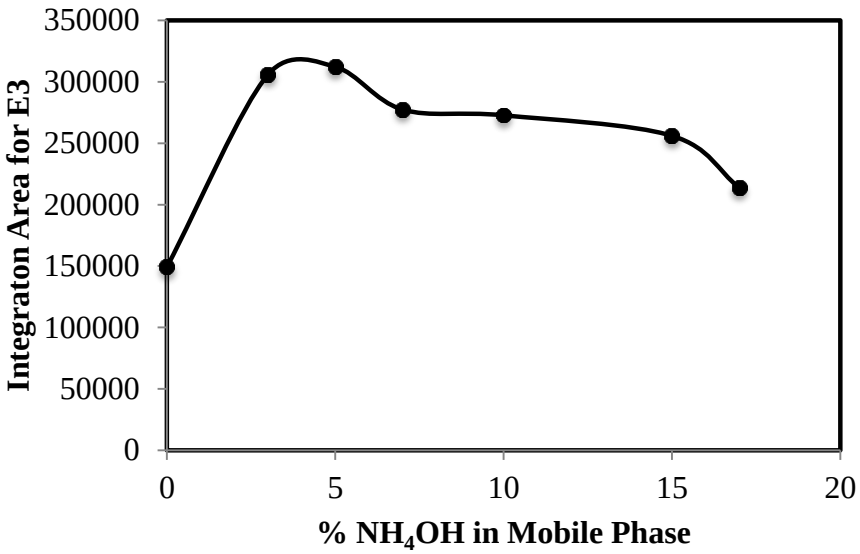


Figure 3.21 Variation of integration area of E3 with % NH_4OH concentration in mobile phase.

3.2.4 Effect of % NH_4OH on Integration Area of EE2

Variance analysis of different NH_4OH concentrations on integration area of EE2 was calculated and depicted in Table 3.21. ANOVA test results indicated that NH_4OH concentration is significant factor for EE2 ionization too. Mean integration area was maximized at NH_4OH = 3%-5% (Figure 3.22) (See appendix Table 7). LSD tests resulted in that there is no significant difference between 3% and 5% in terms of the integrating area (See appendix Table 8). Therefore, the lowest concentration of NH_4OH to get the highest integration area of EE2 was determined as 3%.

Table 3.21 ANOVA for integration area of EE2 at different NH_4OH concentrations.

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3.79E+10	6	6313174006	20.1391802	9.67E-13	2.254053
Within Groups	1.88E+10	60	313477209.7			
Total	5.67E+10	66				

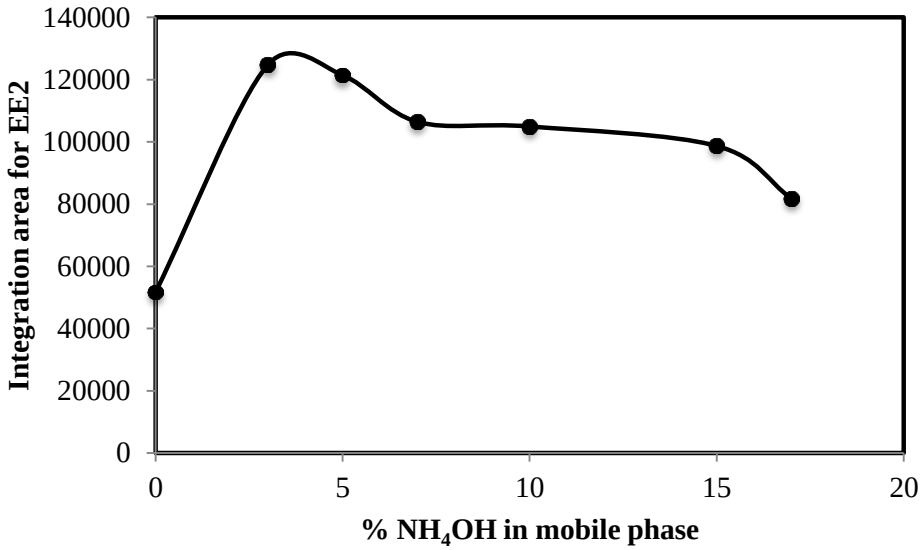


Figure 3.22 Variation of integration area of EE2 with % NH_4OH concentration in mobile phase.

In summary, addition of NH_4OH significantly improved integration area of hormones. Obtaining higher integration area for the same concentration helps

reducing LOD value. Therefore, it can be concluded that NH_4OH has got positive effect on measurement of hormones. Fortunately, 3% NH_4OH addition was optimal and common result for all hormones.

3.3 Determination of Ranges of Operation Parameters for the Measurement of Estrogenic Hormones in LC-MS/MS

The purpose of this study was to select the ranges of the independent instrumental operation variables in the analysis of estrogenic hormones in LC-MS/MS. In other words, the levels of the factors that will be used in optimization of the operating parameters were determined. The factors were sheath gas pressure, spray voltage, vaporizer temperature, aux gas pressure, capillary temperature, ion sweep gas pressure, collision gas pressure and cone position. Single factor experimental design method was used to determine the ranges of factors that significantly affect the response. ANOVA and LSD (Least Significant Difference) test were used for statistical analysis of the results. The response was Integration Area of hormones. The estrogenic hormone concentration was 500 ng/L in mix. Mobile phase was composed of acetonitrile (40%), water (57%) and NH_4OH (200 mM NH_4OH in water) (3%).

3.3.1 Determination of Sheath Gas Pressure Range

The range for sheath gas pressure was selected as SGP= 30 arb and SGP= 40 arb. Three levels; low level, center point and high level were SGP (-)= 30 arb, SPG (center)= 35 arb and SGP (+)= 40 arb, respectively. The other factors were kept constant as collision gas pressure (CGP) = 1.5 m.Torr, capillary temperature (CT) = 278 ^oC, vaporizer temperature (VT) = 258 ^oC, aux gas pressure (AGP) = 20 arb, ion sweep gas pressure (ISGP) = 2.0 arb and spray voltage (SV) = 3500 V (negative mode). The number of replicate was at least n=3 for each estrogenic. Table 3.22 shows the variance analysis for the effect of SGP on integration area of hormones. The statistical analysis clearly stated that SGP significantly affects ($p<0.05$) response area for E1, E2, and E3. However, sheath gas pressure was not a significant factor for

the EE2. Figure 3.23 mean response of replicate at different sheath gas pressures. As seen from the Figure the highest integration area was observed at SGP= 35 arb for E1, E2 and E3.

Although it seems that there was substantial difference in terms of integration area for EE2 at different SGP (Figure 3.24), the mean of 4 replicates indicates no difference in integration area (Table 3.22).

LSD test resulted in that (Table 3.23) most significant differences for integration area for E1, E2 and E3 are between either 30 and 35 arb or 35 and 40 arb. This result means that SGP= 35 arb gives a significantly different integration area compared to that of other SGPs (See appendix Table 9). However, level of SGP does not affect the integration area of EE2. The result of LSD test is in parallel to the results of ANOVA test. In summary, the sheath gas pressure value was determined as SGP= 35 arb for the further experiments of this section.

Table 3.22 ANOVA result for the significance of sheath gas pressure on integration area of hormones in LC-MS/MS.

Source of Variation	*F*	*P-value*	*F critic*	Result
SGP for E1	22.7332669	0.000867	4.737414	Significant
SGP for E2	27.6472086	0.0004756	4.7374141	Significant
SGP for E3	5.30814914	0.0300134	4.2564947	Significant
SGP for EE2	4.13230263	0.0533195	4.2564947	Insignificant

Table 3.23 LSD test for significance of sheath gas pressure levels on integration area of hormones.

Hormone	Levels	$(\overline{Y}_i - \overline{Y}_j)$	t_{critic} $(\alpha .05)$	$t_{calulated} > t_{critic}$ *
	30 vs 35	-7146	3453	Significant
	30 vs 40	2035	3691	Insignificant
E1	35 vs 40	9181	3453	Significant
E2	30 vs 35	-3110	1156	Significant
	30 vs 40	-78	1236	Insignificant
	35 vs 40	3032	1156	Significant
E3	30 vs 35	-2503	2650	Insignificant
	30 vs 40	1245	2650	Insignificant
	35 vs 40	3748	2650	Significant
EE2	30 vs 35	-985	1212	Insignificant
	30 vs 40	-125	1212	Insignificant
	35 vs 40	860	1212	Insignificant

*If $|t_{calculated}| > t_{critic}$, The difference between the means of the levels is significant.

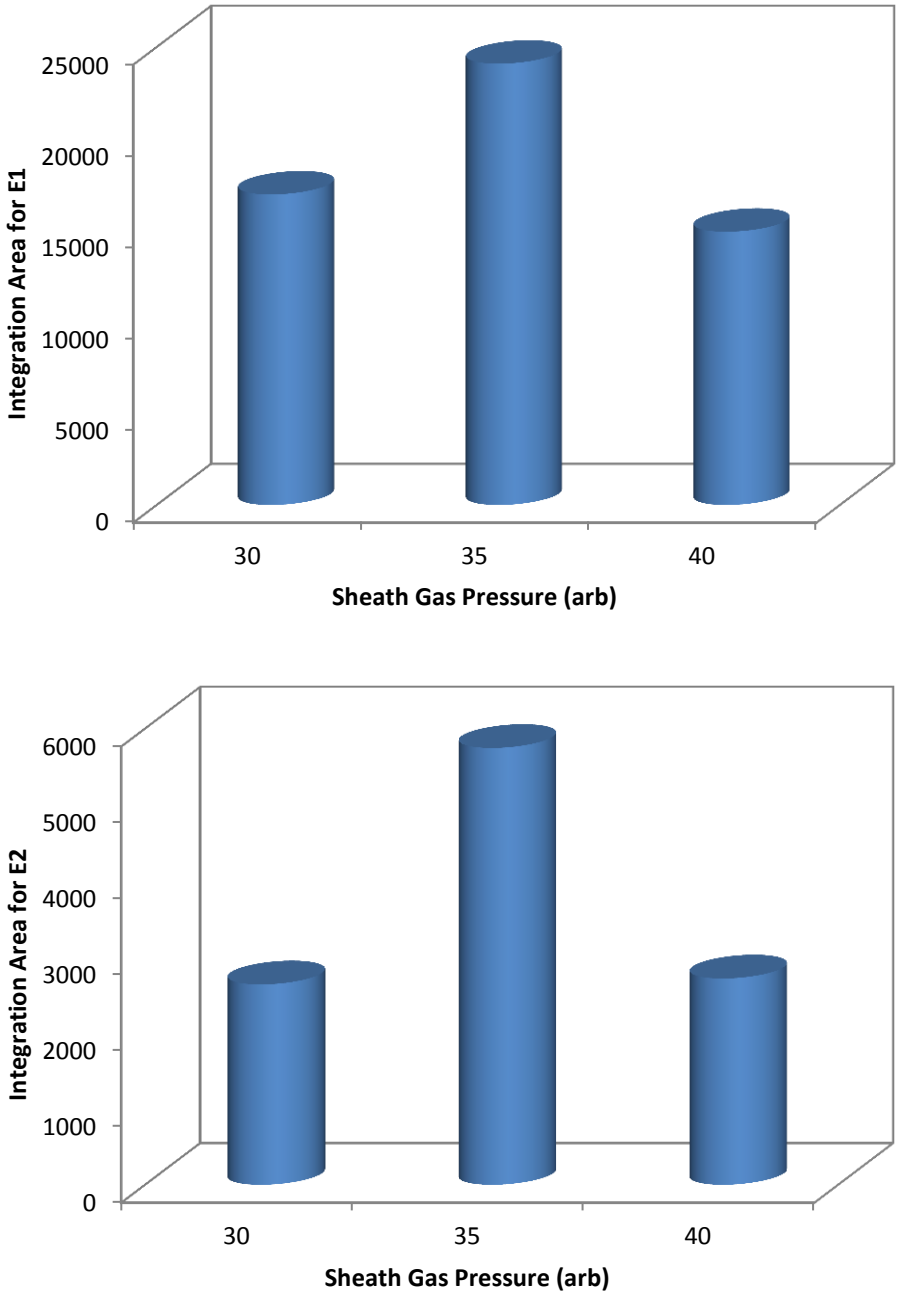


Figure 3.23 Variation of integration area of E1 and E2 with sheath gas pressure.

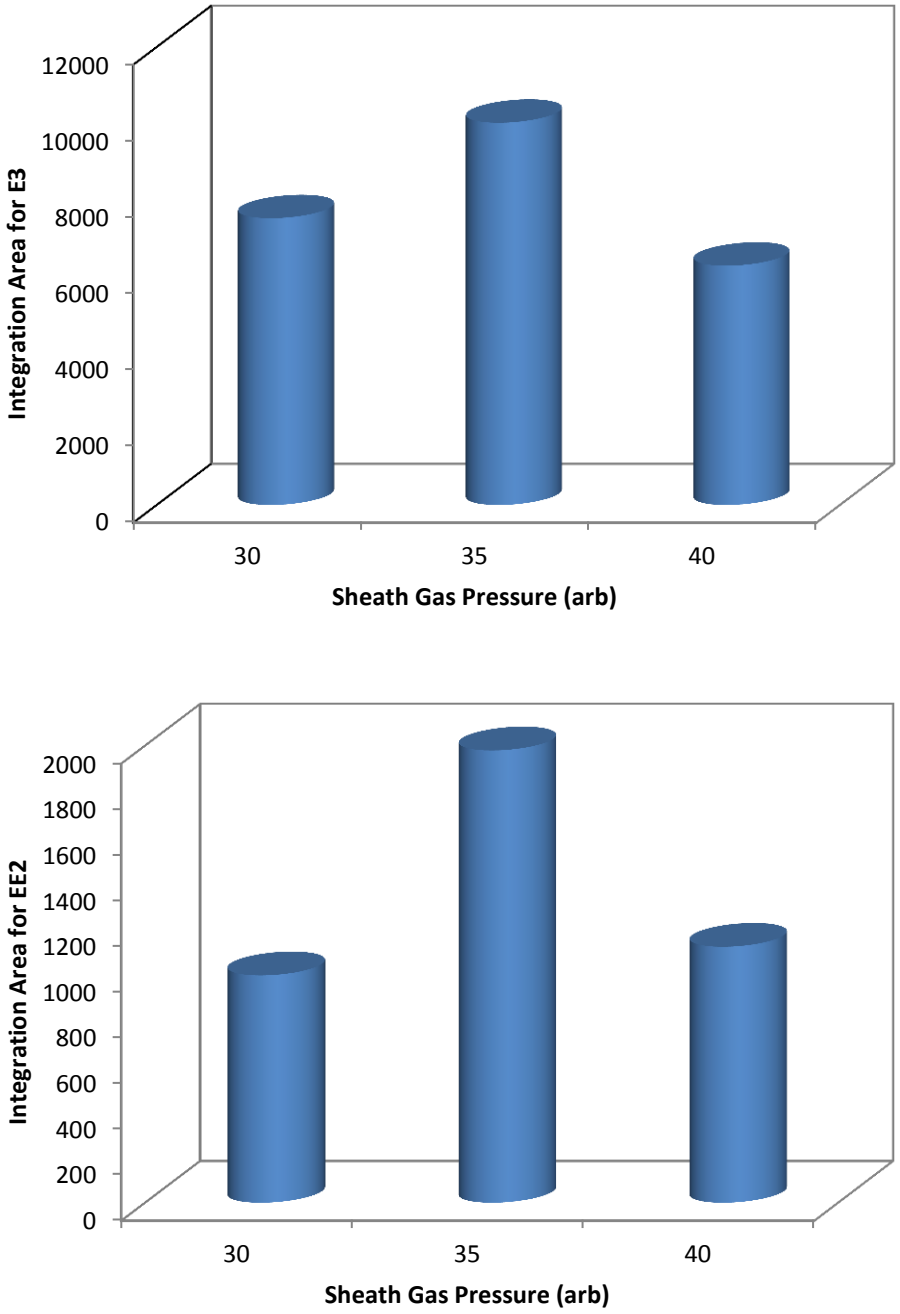


Figure 3.24 Variation of integration area of E3 and EE2 with sheath gas pressure.

3.3.2 Determination of Spray Voltage Range

A wide range spray voltage was available in LC-MS/MS. Therefore, effect of 7 different levels in the range of 2000 V to 3750 V was selected. Single factor with seven levels statistical design method was used to determine if the factor is

significant and then LSD test was applied to determine the most significantly different pairs of levels. In other words, LSD test was used to select the levels which are not significantly different from each other and result in the highest observed integration area for all estrogenic hormones.

ANOVA test showed that spray voltage is a significant factor on integration area of all hormones including EE2 (Table 3.24). Figure 3.25 depicts the variation of integration areas of E1 and E2 for different spray voltages. The high areas were observed at spray voltages between 2250 V and 3250 V for all hormones (See appendix Table 10). LSD test indicated that area obtained for E1 and E2 at SV= 2750 V is significantly higher than the area obtained at either SV= 2250 V and SV= 3250 V (Table 3.25). Similarly, Figure 3.26 depicts the effect of spray voltage on integration area of E3 and EE2. The maximum area obtained as A= 10000 for E3 at SV= 2750 V (See appendix Table 11). LSD test for this hormone gave that the response (integration area) obtained at 2750 V is significantly higher compared to the responses obtained at SV= 3500V and 3750 V. But, it was almost the same as ones observed at SV= 3000V and 3250 V. So, it is not necessary to keep the spray voltage above 2750 V for this hormone.

The highest area for EE2 was achieved at SV= 2250 V. However, LSD test indicated that the area at SV= 2250 V is not significantly different than the areas at spray voltage between SV= 2750 V and SV= 3000V. Although the maximum area was at SV= 2250 V, the spray voltage can be adjusted to SV= 2750 for EE2 (See appendix Tables 19 to 22 for LSD test).

In summary, the most suitable spray voltage was determined as SV= 2750 V for all hormones.

Table 3.24 ANOVA result for the significance of spray voltage on integration area of hormones in LC- MS/MS.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F critic*	Result
E1	1.23E+09	6	205124388.6	11.4733787	1.83E-06	2.445259	Significant
E2	73190514	6	12198419.02	7.9685823	4.53E-05	2.445259	Significant
E3	1.62E+08	6	26977191.66	9.04536432	1.56E-05	2.445259	Significant
EE2	10075369	6	1679228.181	4.53229833	0.002496	2.445259	Significant

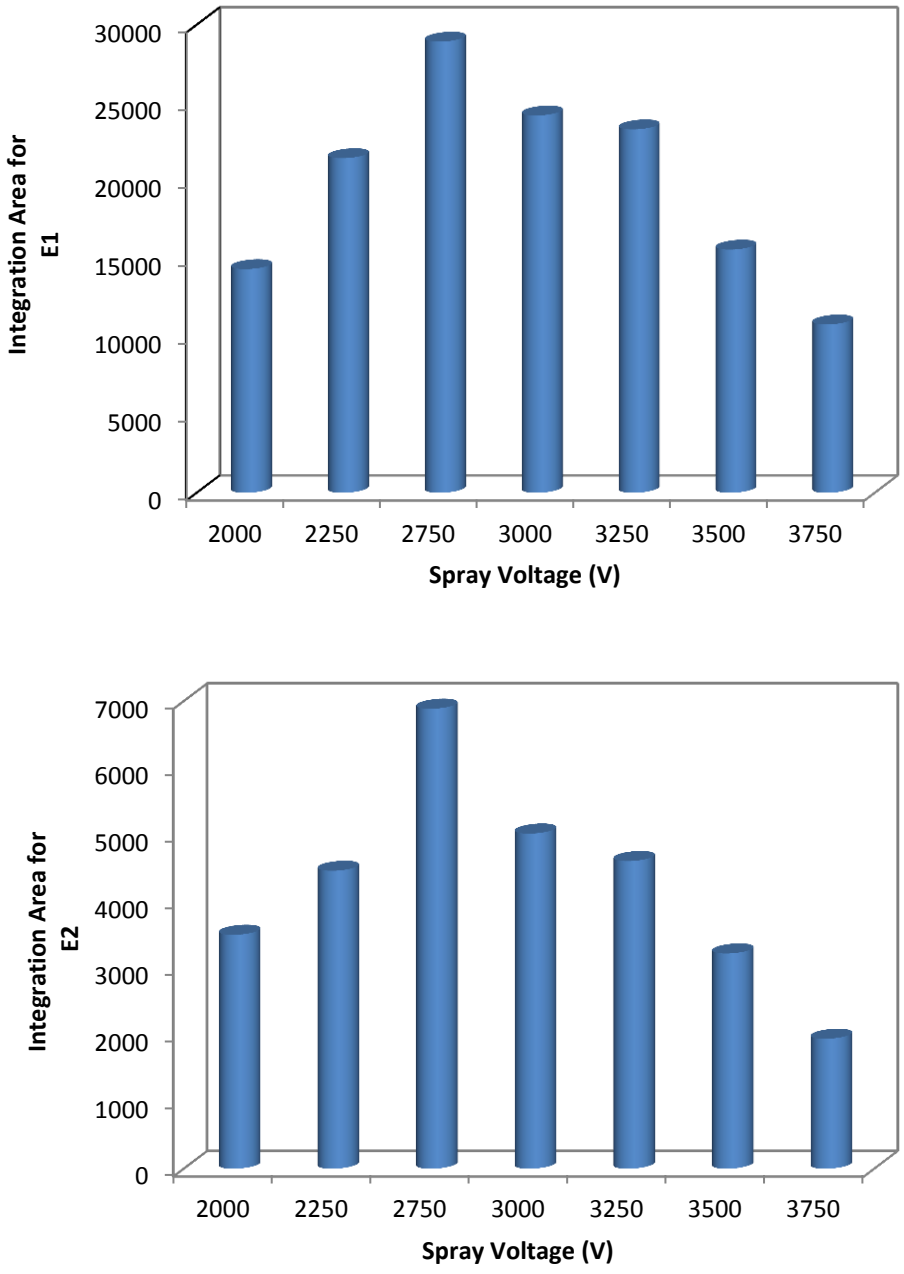


Figure 3.25 Variation of integration area of E1 and E2 with spray voltages.

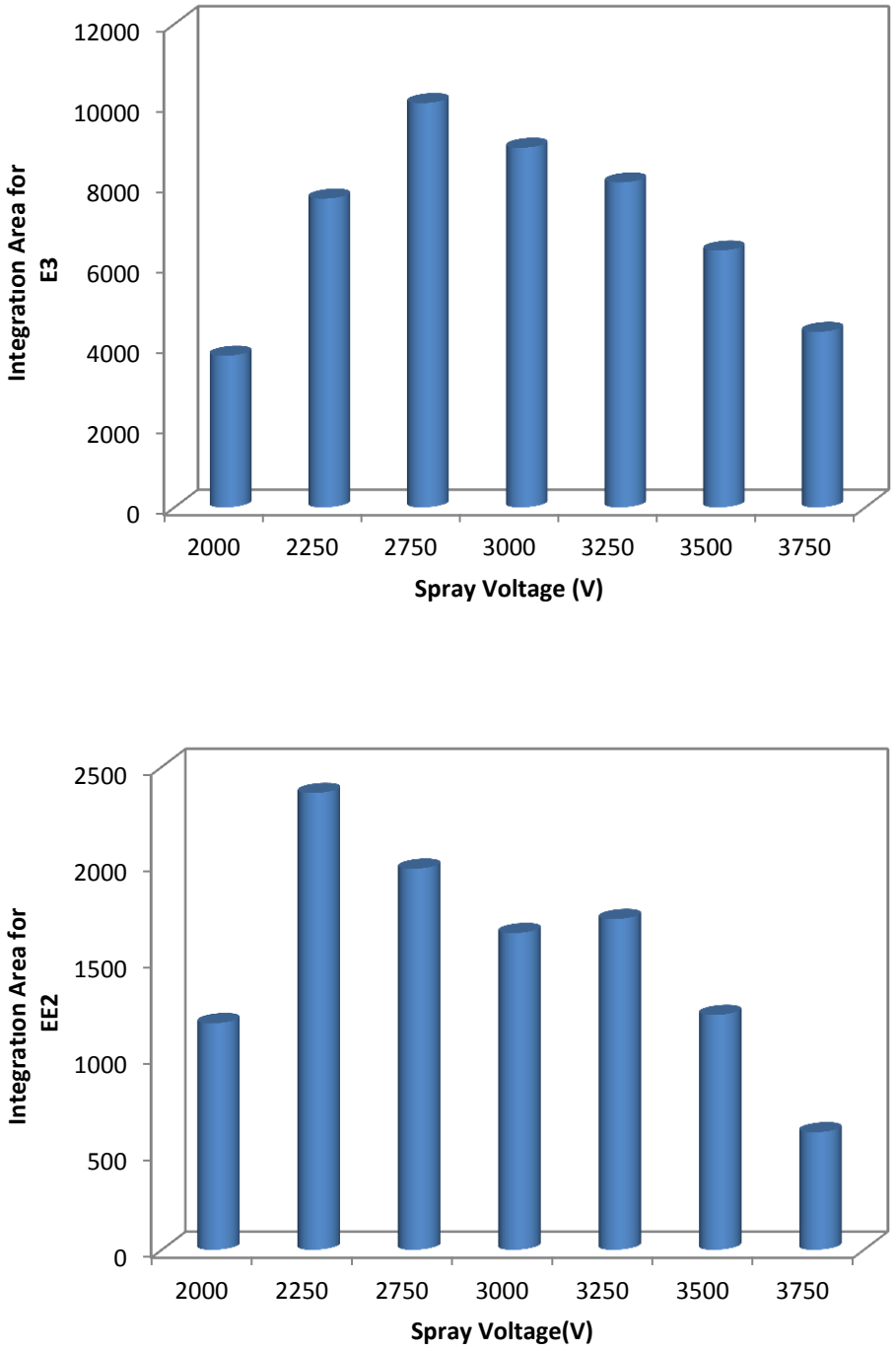


Figure 3.26 Variation of integration area of E3 and EE2 with spray voltages.

Table 3.25 LSD test results for significance of spray voltage levels on integration area of hormones.

Type of hormone	Levels	$(\overline{Y}_i - \overline{Y}_j)$ $t_{calculated}$	t_{critic} (α .05)	$t_{calulated} > t_{critic}$ *
E1	2750 vs 3000	4732	5476.7502	Insignificant
	2750 vs 3250	5624	5476.7502	Significant
	2750 vs 3500	13332	5476.7502	Significant
	2750 vs 3750	18131	5476.7502	Significant
E2	2750 vs 3000	1869	1603	Significant
	2750 vs 3250	2280	1603	Significant
	2750 vs 3500	3664	1603	Significant
	2750 vs 3750	4942	1603	Significant
E3	2750 vs 3000	1109	2237	Insignificant
	2750 vs 3250	1973	2237	Insignificant
	2750 vs 3500	3663	2237	Significant
	2750 vs 3750	5682	2237	Significant
EE2	2250 vs 2750	392	788	Insignificant
	2250 vs 3000	726	788	Insignificant
	2250 vs 3250	653	788	Insignificant
	2250 vs 3500	1150	788	Significant
	2250 vs 3750	1757	788	Significant

*If $|t_{calculated}| > t_{critic}$, The difference between the means of the levels is significant.

3.3.3 Determination of Vaporizer Temperature Range

Vaporizer temperature was the third factor to be investigated. The range was between 200^{o}C and 400^{o}C. Number of level was 8 and the number of replicate was 5 at each level for all hormones. Spray voltage and sheath gas pressure were SV= 2750 V and SGP= 35 arb as determined in the previous experiments. The result of ANOVA test indicated that area of all hormones can change significantly as vaporizer temperature is changed (Table 3.26). In other words, levels of vaporizer temperature significantly affect the integration area.

Figure 3.27 depicts the variation of integration area of E1 and E2 at different vaporizer temperatures. The maximum area was obtained as A= 60000 at VT= 375 ^{o}C for E1 (See appendix Table 12). LSD test resulted in that this values are significantly higher than the value that can be obtained at VT= 400^{o}C at which the second highest area was observed (Table 3.27). In the case of E2, the area was the highest (A= 8000) at 375 ^{o}C, but the next closest area were observed at VT= 350 ^{o}C and 400^{o}C. However, LSD test indicated that the area obtained at 375^{o}C is significantly higher than that of at VT= 350^{o}C, but the area difference for VT= 375 ^{o}C and VT= 400^{o}C are not significantly different meaning that the system can be operated at VT= 375 ^{o}C for E2.

The situation was almost the same for E3. The two close integration areas were observed at VT= 375 ^{o}C and 400^{o}C (See appendix Table 13). But, it was statistically proved that area at 375^{o}C is significantly higher than the area obtained at 400 ^{o}C. Similarly, the highest area was achieved at VT=375^{o}C for EE2 (Figure 3.28). Although, the values for VT= 375^{o}C and 400^{o}C are not statistically different, it is practical to operate the LC-MS/MS at around VT= 375 ^{o}C. As a result, VT was selected as VT= 375 ^{o}C for further optimization studies. (See appendix Tables 23 to 26 for more information about LSD test.)

Table 3.26 ANOVA result for the significance of vaporizer temperature on integration area of hormones in LC- MS/MS.

Type of hormone	*SS*	*df*	*MS*	*F*	*P-value*	*F critic*	Result
E1	7147465552	7	1021066507	17	0	2	Significant
E2	68613868.75	7	9801981.25	5.19	0.00	2.31	Significant
E3	1397643157	7	199663308.1	26.92370718	1.072E-11	2.3127412	Significant
EE2	32427791.15	7	4632541.593	9.850909004	1.709E-06	2.3127412	Significant

Table 3.27 LSD test results for significance of vaporizer temperature level on integration area of hormones.

Type of Hormone	Levels	$(\overline{Y}_i - \overline{Y}_j)$ $t_{calculated}$	t_{critic} (α .05)	$t_{calulated} > t_{critic}$ *
E1	375 vs 400	11342	9853	Significant
	375 vs 425	28183	9853	Significant
	375 vs 450	23018	9853	Significant
E2	375 vs 300	-2878.64	1771.15	Significant
	375 vs 400	563.16	1771.15	Insignificant
	375 vs 425	1818.42	1771.15	Significant
	375 vs 450	2704.19	1771.15	Significant
E3	375 vs 400	3880	3510	Significant
	375 vs 425	10056	3510	Significant
	375 vs 450	10170	3510	Significant
EE2	375 vs300	-963	884	Significant
	375 vs 400	675	884	Insignificant
	375 vs 425	1335	884	Significant
	375 vs 450	1985	884	Significant

*If $|t_{calculated}| > t_{critic}$, The difference between the means of the levels is significant.

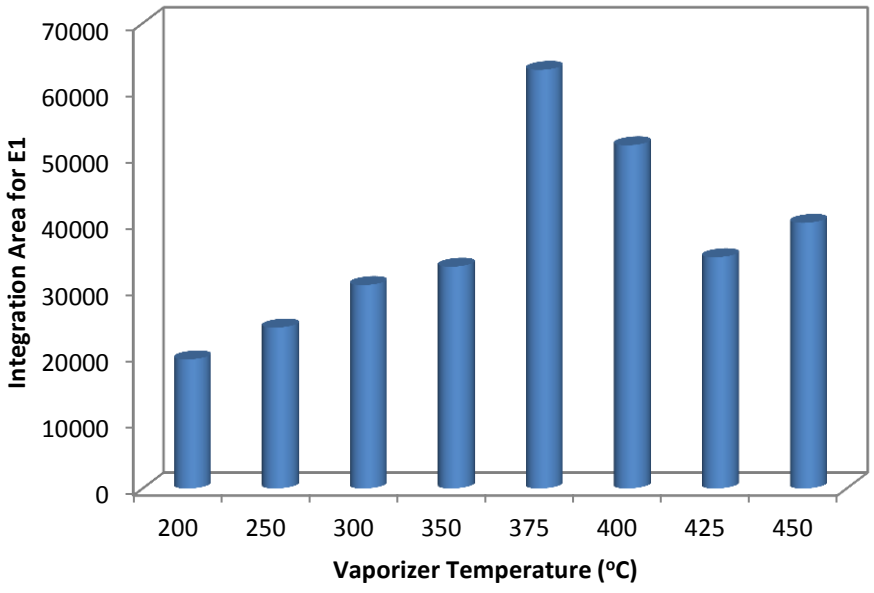


Figure 3.27 Variation of integration area of E1 and E2 with vaporizer temperature.

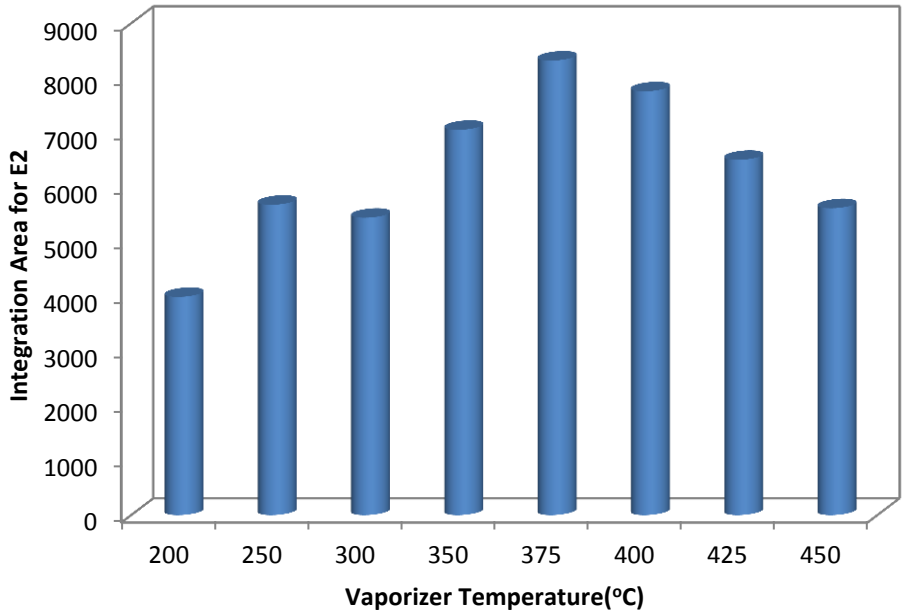


Figure 3.27 Variation of integration area of E1 and E2 with vaporizer temperature (continued).

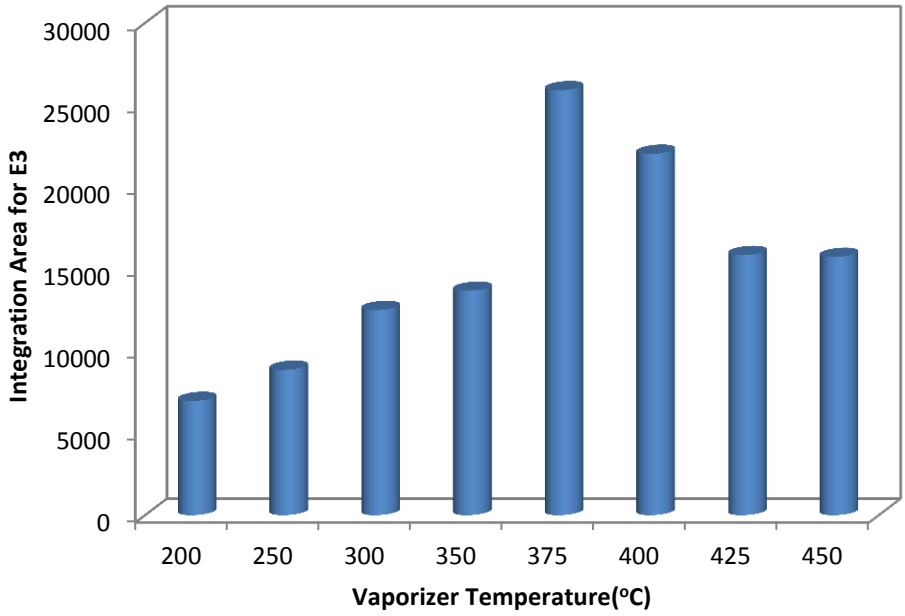


Figure 3.28 Variation of integration area of E3 and EE2 with vaporizer temperature.

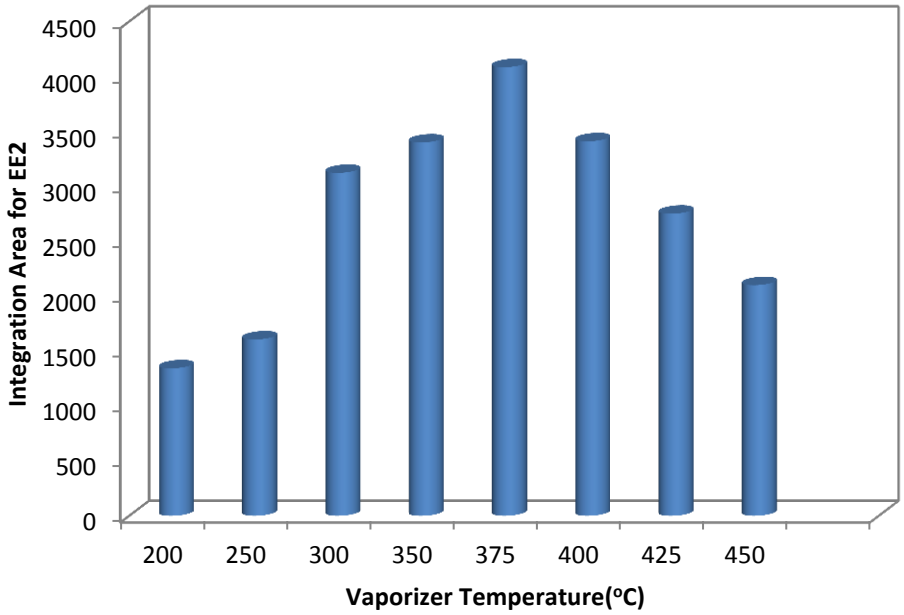


Figure 3.28 Variation of integration area of E3 and EE2 with vaporizer temperature (continued).

3.3.4 Determination of Aux Gas Pressure Range

Aux gas pressure (AGP) was varied between AGP= 5 arb to 20 arb. The number of level and replicate were 4 and 5, respectively. Spray gas voltage, vaporizer temperature and sheath gas pressure was kept constant at the value determined in the previous sections. ANOVA analysis indicated that there is no significant difference in integration area of all hormones when different aux gas pressures were used (Table 3.28), (See appendix Table 14). Integration area of hormones at different aux gas pressures were depicted in Figures from 3.29 to 3.30. Although it seems that there is some differences for different aux gas pressures, LSD test indicated that the difference between the integration areas of all hormones at different aux gas pressures is insignificant (Table 3.29). That means LC-MS/MS can be operated at any aux gas pressures and it was selected as AGP= 20 arb.

Table 3.28 ANOVA result for the significance of aux gas pressure on integration area of hormones in LC-MS/MS.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F critic*	Result
E1	33102504.01	3	11034168	2.149427173	0.1340306	5.2922141	Insignificant
E2	3661613.117	3	1220537.706	0.427232238	0.7362075	3.2388715	Insignificant
E3	28215116.44	3	9405038.812	1.79175616	0.1892729	3.2388715	Insignificant
EE2	1418437.354	3	472812.4515	0.298444152	0.8260087	3.2388715	Insignificant

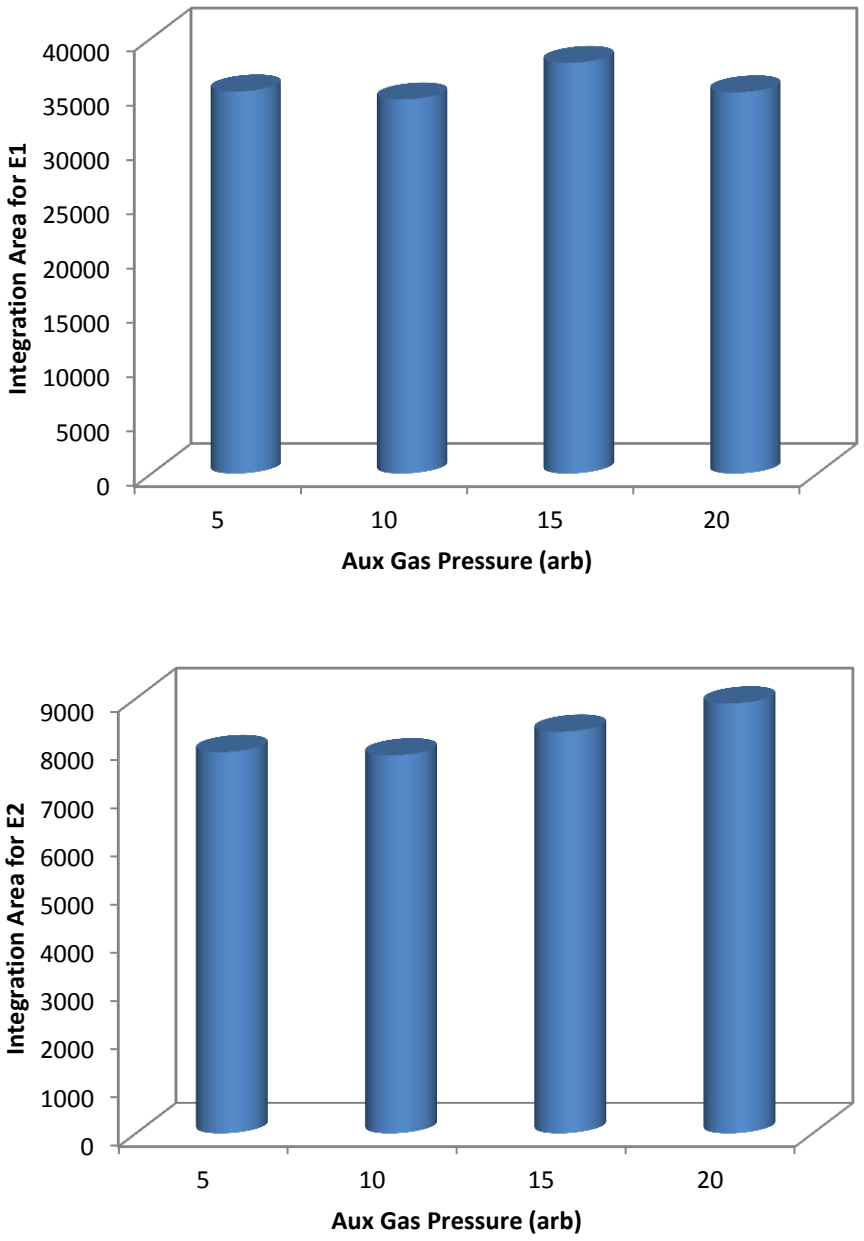


Figure 3.29 Variation of integration area of E1 and E2 with aux gas pressure.

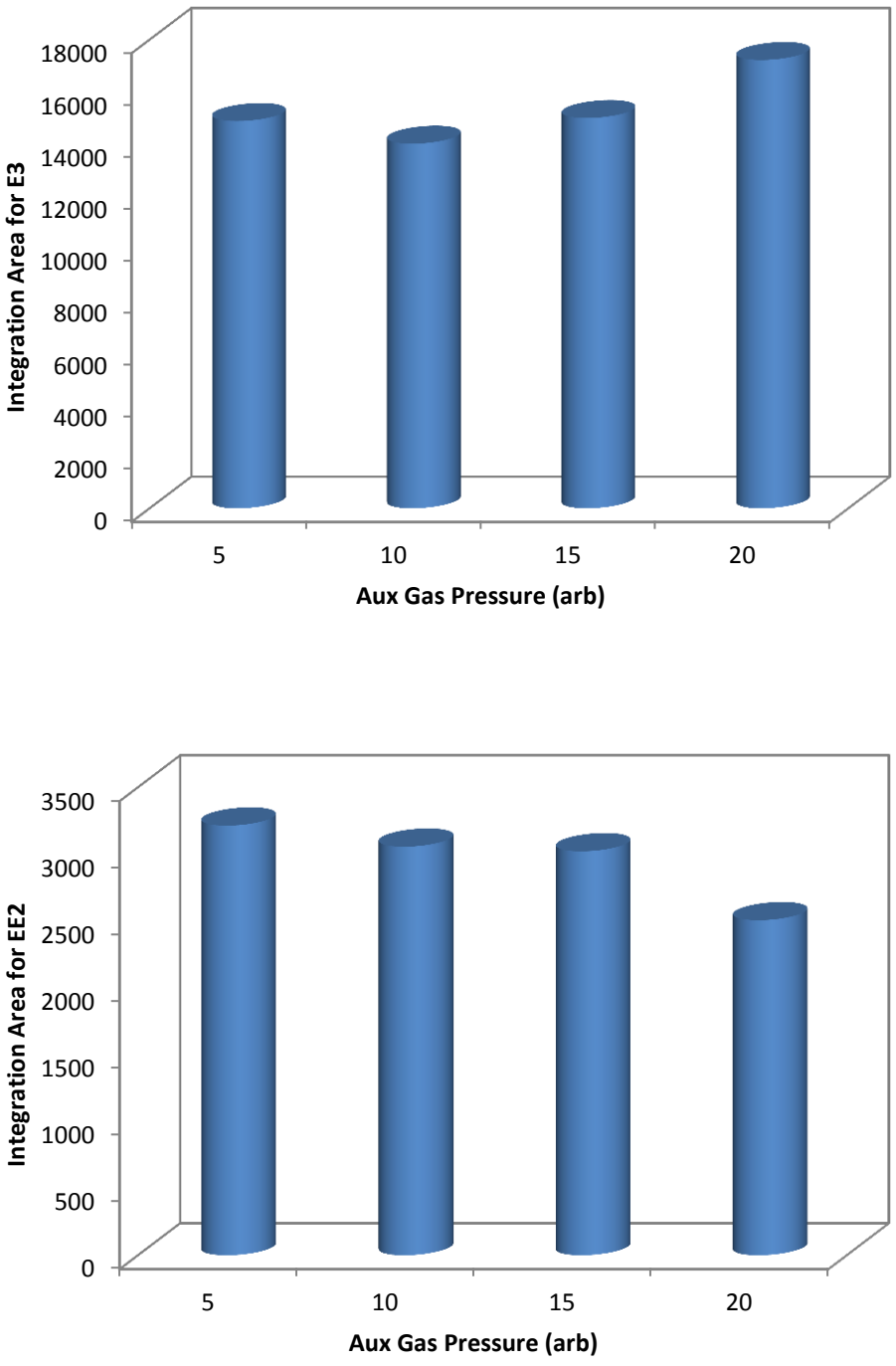


Figure 3.30 Variation of integration area of E3 and EE2 with aux gas pressure.

Table 3.29 LSD test results for significance of aux gas pressure levels on integration area of hormones.

Type of hormone	Yi vs Yj	$(\overline{Y_i} - \overline{Y_j})$ $t_{calculated}$	t_{critic} (α .05)	$t_{calulated} > t_{critic}$ *
E1	5 vs 10	736	4186	Insignificant
	5 vs 15	-2625	4186	Insignificant
	5 vs 20	81	4186	Insignificant
	10 vs 15	-3361	4186	Insignificant
	10 vs 20	-655	4186	Insignificant
	15 vs 20	2706	4186	Insignificant
E2	5 vs 10	68	2266	Insignificant
	5 vs 15	-422	2266	Insignificant
	5 vs 20	-1006	2266	Insignificant
	10 vs 15	-490	2266	Insignificant
	10 vs 20	-1074	2266	Insignificant
	15 vs 20	-584	2266	Insignificant
E3	5 vs 10	871	4233	Insignificant
	5 vs 15	-115	4233	Insignificant
	5 vs 20	-2346	4233	Insignificant
	10 vs 15	-986	4233	Insignificant
	10 vs 20	-3217	4233	Insignificant
	15 vs 20	-2231	4233	Insignificant
EE3	5 vs 10	158	1688	Insignificant
	5 vs 15	192	1688	Insignificant
	5 vs 20	709	1688	Insignificant
	10 vs 15	34	1688	Insignificant
	10 vs 20	551	1688	Insignificant
	15 vs 20	516	1688	Insignificant

*If $| t_{calculated} | > t_{critic}$, The difference between the means of the levels is significant.

3.3.5 Determination of Capillary Temperature Range

A narrow range was selected for capillary temperature (CT). It was between CT=300 ^{o}C and 350 ^{o}C. ANOVA test indicated that capillary temperature levels haven't got significant effect on integration area of E1, E3 and EE2. But it is significant for E2 (Table 3.30). Integration area for E1 was A= 45000 at CT= 300 ^{o}C but slightly decreased to around A= 35000 at CT= 350 ^{o}C (Figure 3.31). According to this result, it can be concluded increasing temperature adversely affect the

integration area (See appendix Table 15). However, LSD test statistically proof that the decrease in the area with the increase in temperature is not significant (Table 3.31). The similar effect can be observed for E3 and EE2 (Figure 3.32). But LSD test results indicated that no significant difference for the integration area of these hormones. Finally, E2 is affected by the levels of capillary temperature (Table 3.30), the highest area was obtained at CT= 300^{o}C and that area was significantly different than ones obtained at CT= 325 ^{o}C and 350 ^{o}C. Capillary temperature was selected as CT= 300^{o}C for the benefit of E2.

Table 3.30 ANOVA result for the significance of capillary temperature on integration area of hormones in LC-MS/MS.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F critic*	Result
E1	187633558	2	93816778.98	3.132859771	0,0927606	4,2564947	Insignificant
E2	27995798.9	2	13997899.45	5.077129113	0.0334116	4.2564947	Significant
E3	18298921.33	2	9149460.664	1.110225863	0.3707189	4.2564947	Insignificant
EE2	3414233.999	2	1707117	2.895340953	0.106941	4.2564947	Insignificant

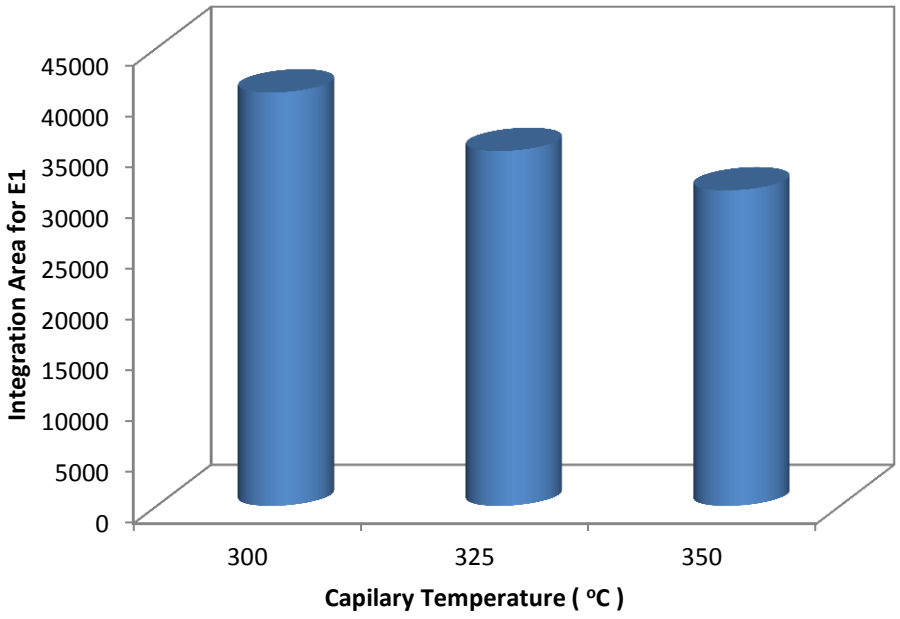


Figure 3.31 Variation of integration area of E1 and E2 with capillary temperature.

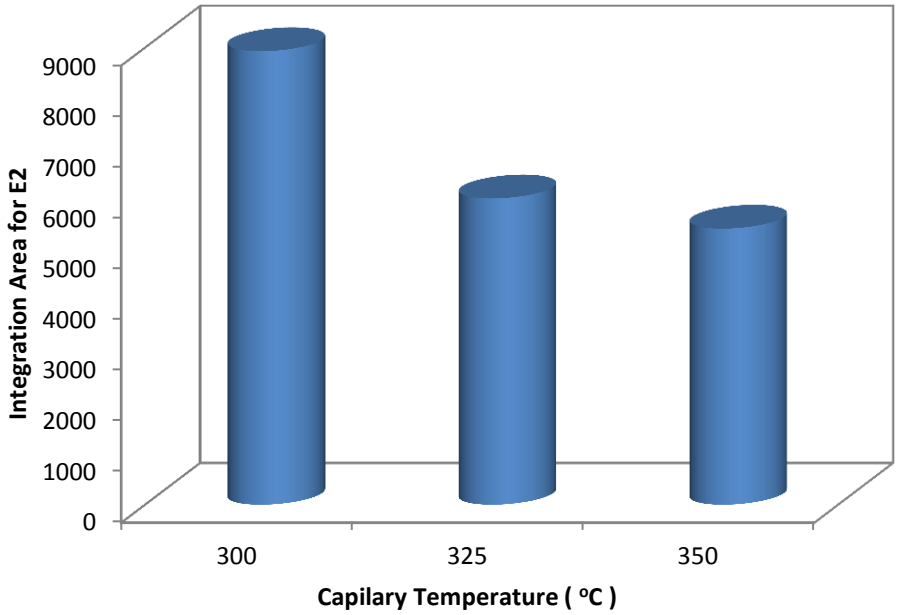


Figure 3.31 Variation of integration area of E1 and E2 with capillary temperature (continued).

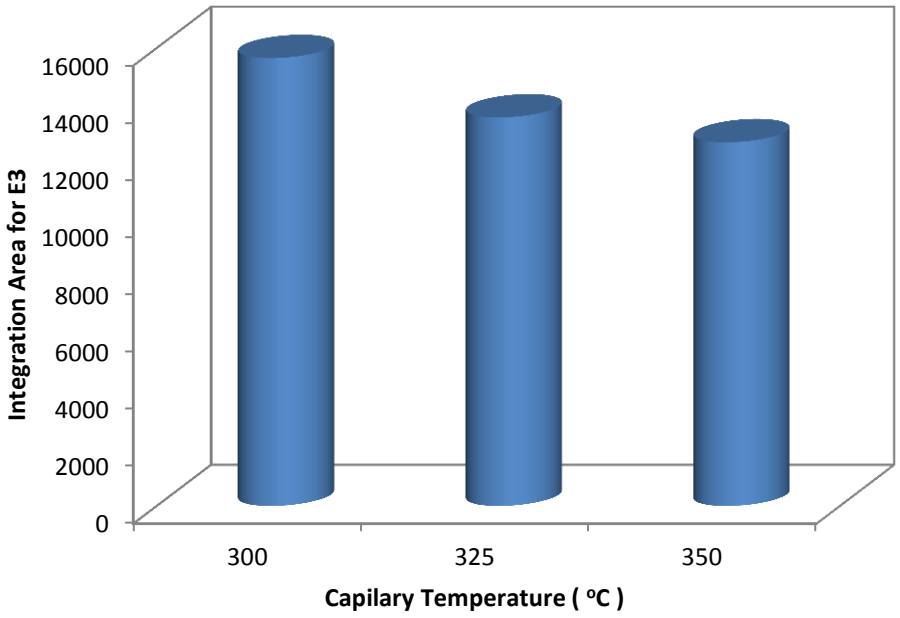


Figure 3.32 Variation of integration area of E3and EE2 with capillary temperature.

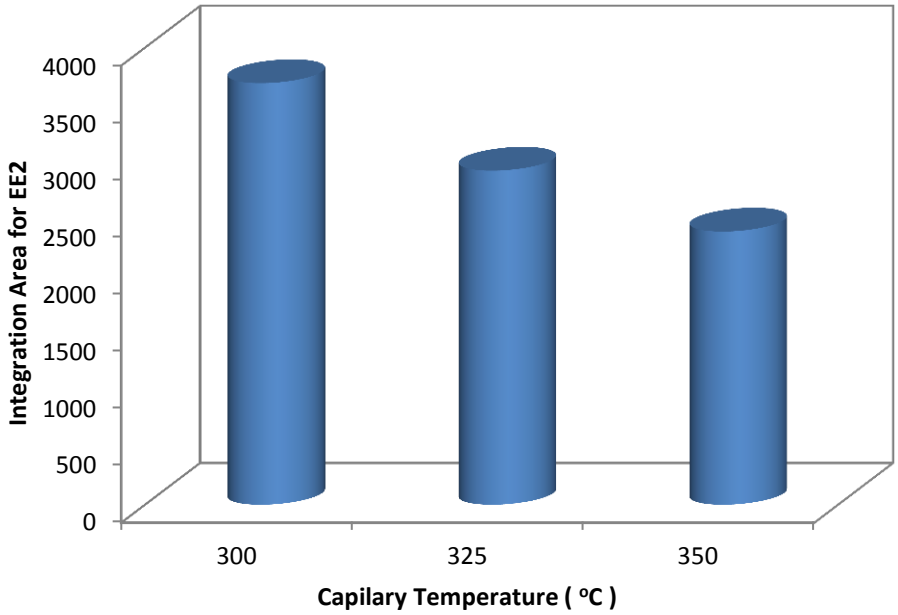


Figure 3.32 Variation of integration area of E3and EE2 with capillary temperature (continued).

Table 3.31 LSD test results for significance of capillary temperature levels on integration area of hormones.

Type of hormone	Levels	$(\overline{Y}_i - \overline{Y}_j)$ $t_{calculated}$	t_{critic} $(\alpha\ .05)$	$t_{calulated} > t_{critic}$ *
E1	300 vs 325	5756	12963	Insignificant
	300 vs 350	9624	12963	Insignificant
	325 vs 350	3869	12963	Insignificant
E2	300 vs 325	2902	2656	Significant
	300 vs 350	3496	2656	Significant
	325 vs 350	594	2656	Insignificant
E3	300 vs 325	2074	4592	Insignificant
	300 vs 350	2944	4592	Insignificant
	325 vs 350	869	4592	Insignificant
EE2	300 vs 325	767	1765	Insignificant
	300 vs 350	1300	1765	Insignificant
	325 vs 350	533	1765	Insignificant

*If $|t_{calculated}| > t_{critic}$, The difference between the means of the levels is significant.

3.3.6 Determination of Ion Sweep Gas Pressure Range

The experiments at different ion sweep gas pressures (ISGP) resulted in that this factor is not significant (Table 3.32). Even at different levels of these factors no substantial difference was observed for integration area (See appendix Table 16). As seen from Figures 3.33 and 3.34, the areas are very close to each other. LSD test statistically proves that changing sweep gas pressure does not affect the integration area (Table 3.33). Therefore, SGP was selected as SGP= 0 arb for the further experiments.

Table 3.32 ANOVA result for the significance of ion sweep gas pressure on integration area of hormones in LC-MS/MS.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F critic*	Result
E1	7173249.081	2	3586624.541	0.498534537	0.6232527	4.2564947	Insignificant
E2	5268792.042	2	2634396.021	1.881017459	0.2077072	4.2564947	Insignificant
E3	1625143.081	2	812571.5405	0.174265694	0.8428417	4.2564947	Insignificant
EE2	3611353.8	2	1805676.9	3.586679528	0.0715303	4.2564947	Insignificant

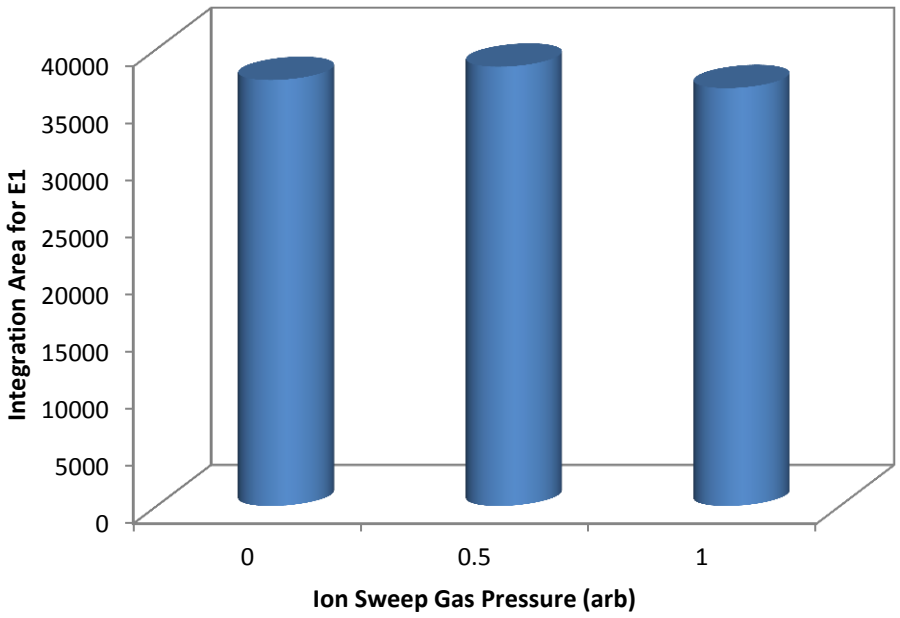


Figure 3.33 Variation of integration area of E1 and E2 with variable ion sweep gas pressure.

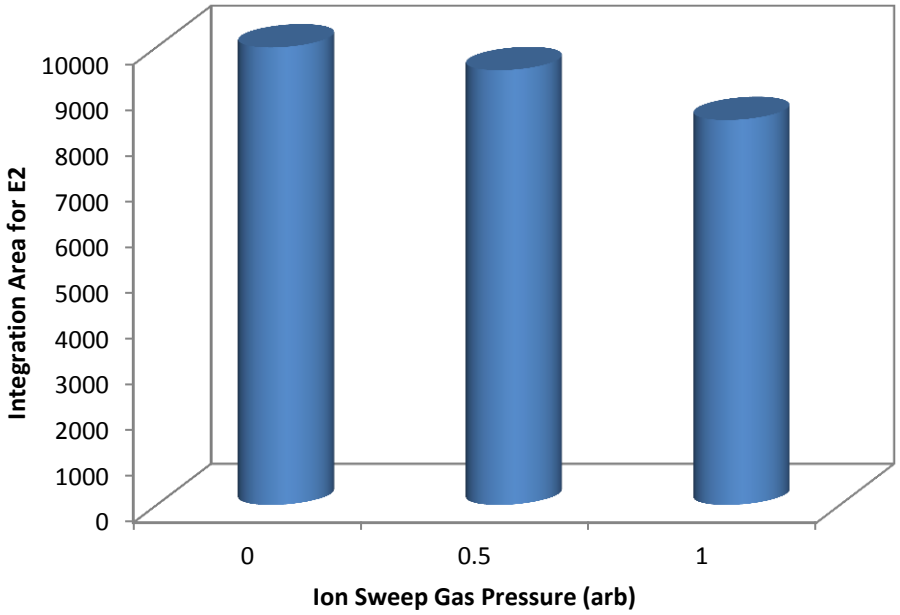


Figure 3.33 Variation of integration area of E1 and E2 with variable ion sweep gas pressure (continued).

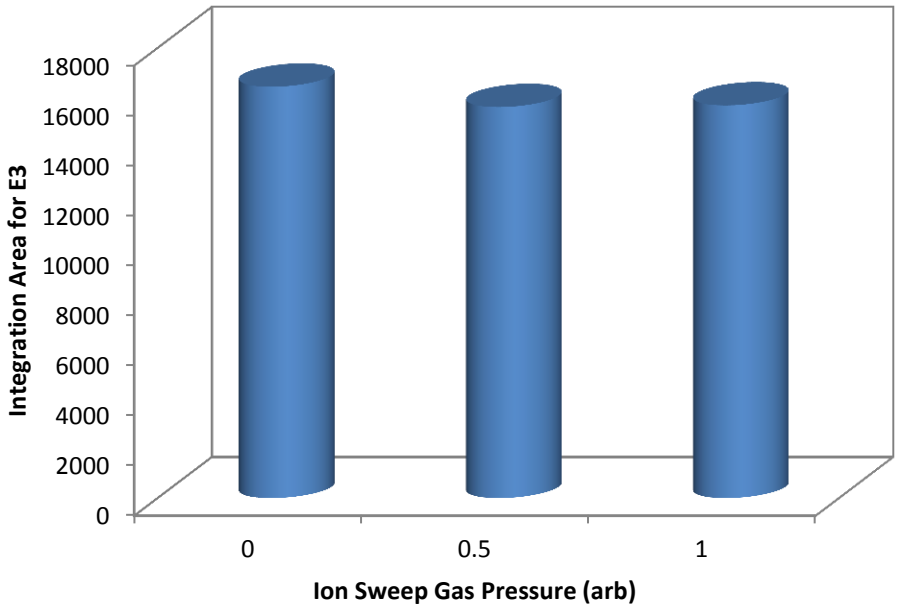


Figure 3.34 Variation of integration area of E3 and EE2 with variable ion sweep gas pressure.

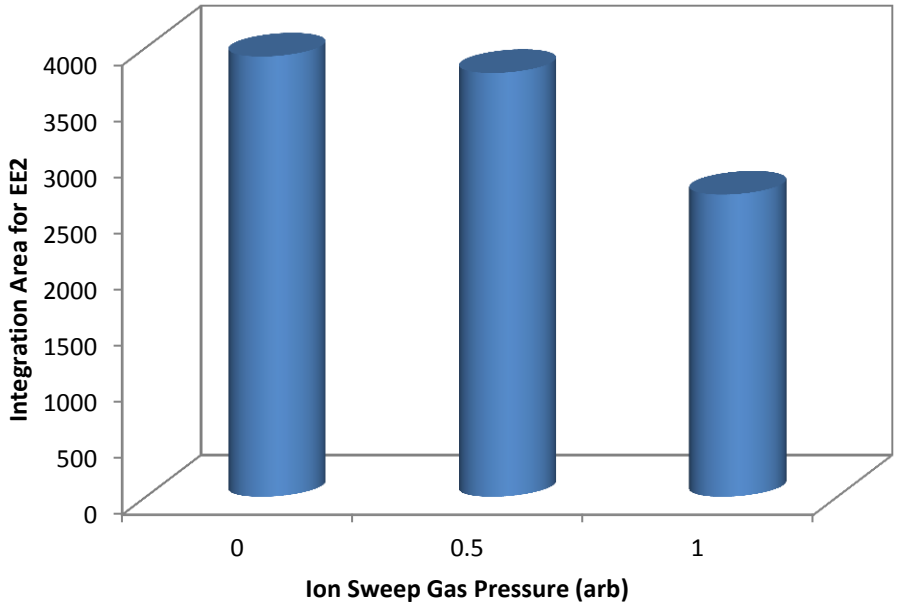


Figure 3.34 Variation of integration area of E3 and EE2 with variable ion sweep gas pressure (continued).

Table 3.33 LSD test results for significance of ion sweep gas pressure levels on integration area of hormones.

Type of hormone	Levels	$(\overline{Y}_i - \overline{Y}_j)$ $t_{calculated}$	t_{critic} (α .05)	$t_{calulated} > t_{critic}$ *
E1	0.5 vs 0	1166	4290	Insignificant
	0.5 vs 1	1875	4290	Insignificant
	0 vs 1	709	4290	Insignificant
E2	0.5 vs 0	-497	1893	Insignificant
	0.5 vs 1	1089	1893	Insignificant
	0 vs 1	1587	1893	Insignificant
E3	0.5 vs 0	-812	3454	Insignificant
	0.5 vs 1	-67	3454	Insignificant
	0 vs 1	745	3454	Insignificant
EE2	0.5 vs 0	-147	1631	Insignificant
	0.5 vs 1	1083	1631	Insignificant
	0 vs 1	1230	1631	Insignificant

*If $|t_{calculated}| > t_{critic}$, The difference between the means of the levels is significant.

3.3.7 Range Determination for Cone Position

There are 3 positions on the cone. These are B, C and D. B position increases the distance between ionization point and sample injection point to ionization chamber. This distance can sometimes affect the amount of sample that enters to the ionization chamber. Therefore, this factor was considered as important to get better integration area. ANOVA test results indicated that cone position is a significant factor for integration area of E1, E2 and E3 (Table 3.34). The general trend of integration area for the different cone position is the increase in area as cone position was changed from B to D for these hormones (Figures 3.35 and 3.36). In other words, the highest area was observed at D position (See appendix Table 17). LSD test results depicts that there are significant differences between B position with respect to C and D positions. But, the difference is not significant for C and D positions.

The situation is quite different for EE2. Cone position is not significant and although there is a slight increase in the area as position was changed from B to D (Figure 3.36), this increase is not statistically significant (Table 3.35). In summary, D position was determined as the best.

Table 3.34 ANOVA result for the significance of cone position on integration area of hormones in LC-MS/MS.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F critic*	Result
E1	526328775.2	2	263164387.6	28.28421128	0.0001315	4.2564947	Significant
E2	30813020.48	2	15406510.24	17.18222307	0.0008453	4.2564947	Significant
E3	170141837.9	2	85070918.93	47.2213957	1.69E-05	4.2564947	Significant
EE2	2269094.62	2	1134547.31	1.913252066	0.2030504	4.2564947	Insignificant

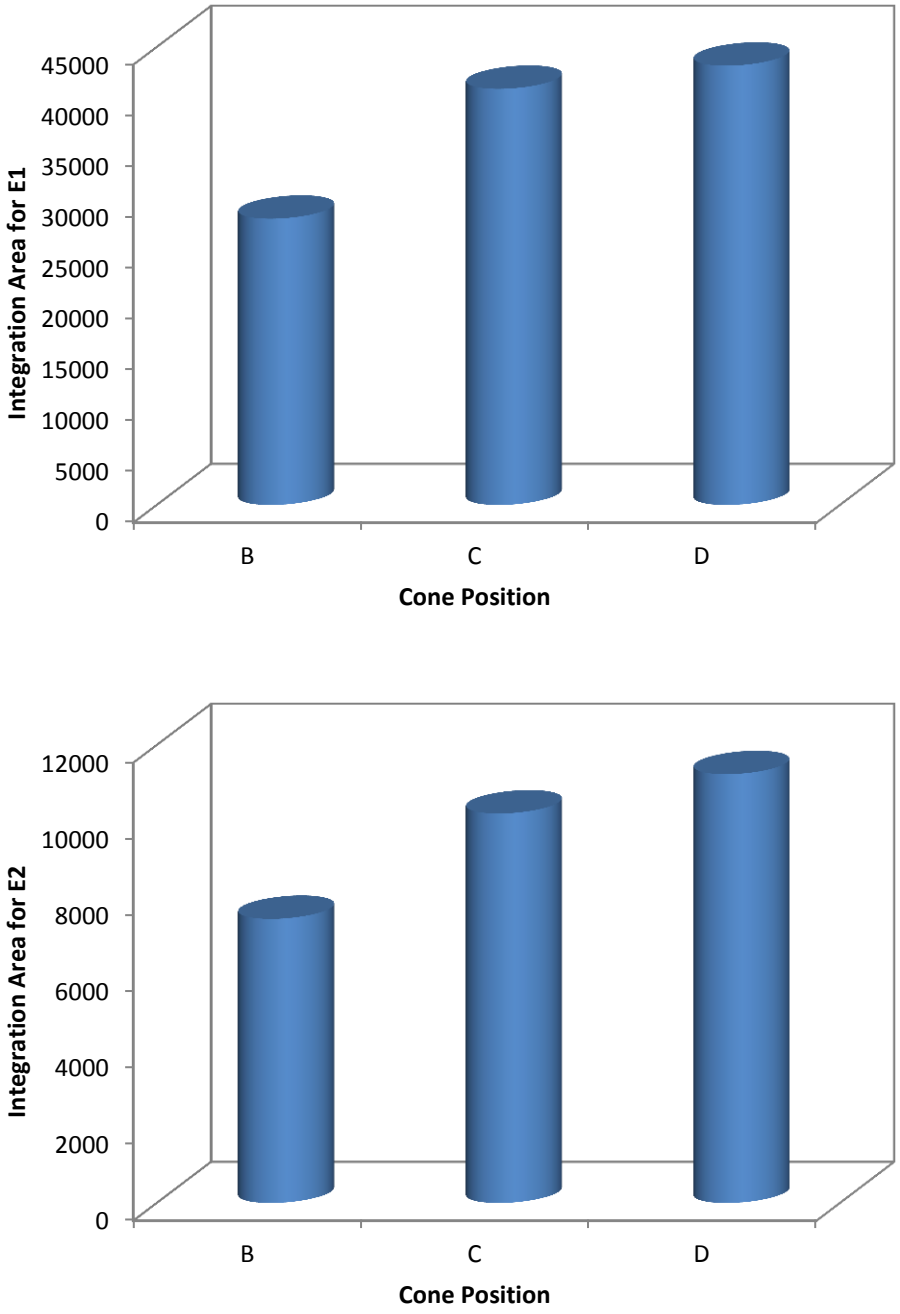
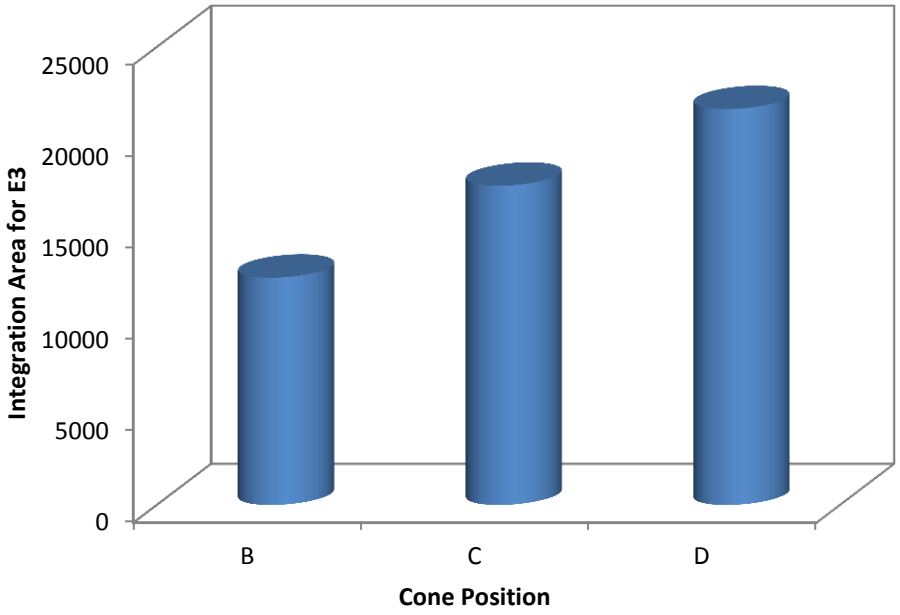


Figure 3.35 Variation of integration area of E1 and E2with cone position.



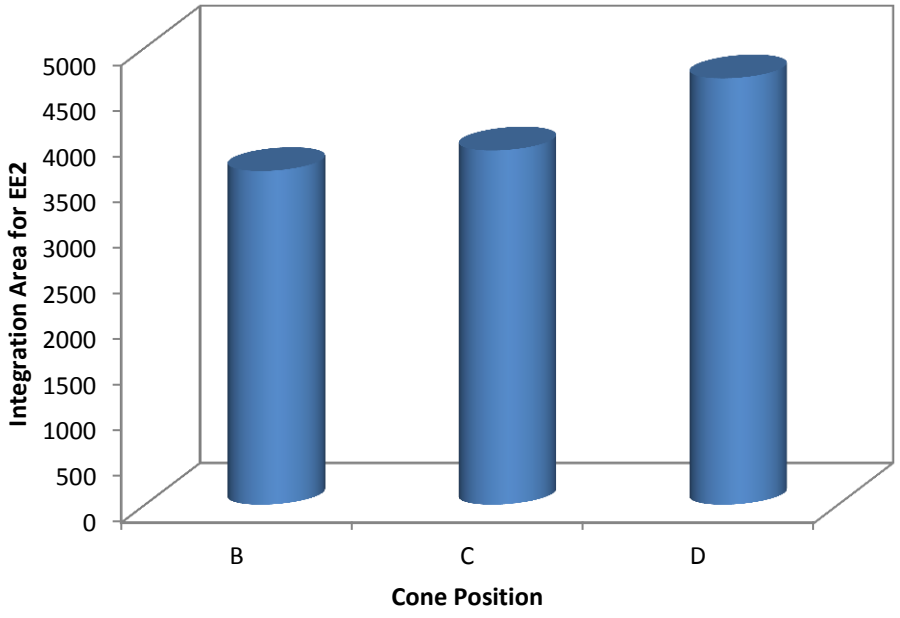


Figure 3.36 Variation of integration area of E3 and EE2 with cone position.

Table 3.35 LSD test results for significance of cone position levels on integration area of hormones.

Type of hormone	Levels	$(\overline{Y}_i - \overline{Y}_j)$ $t_{calculated}$	t_{critic} (α .05)	$t_{calulated} > t_{critic}$ *
E1	B vs C	-12770	4879	Significant
	B vs D	-15049	4879	Significant
	C vs D	-2279	4879	Insignificant
E2	B vs C	-2764	1515	Significant
	B vs D	-3795	1515	Significant
	C vs D	-1031	1515	Insignificant
E3	B vs C	-5025	2147	Significant
	B vs D	-9211	2147	Significant
	C vs D	-4186	2147	Significant
EE2	B vs C	-225	1232	Insignificant
	B vs D	-1014	1232	Insignificant
	C vs D	-789	1232	Insignificant

*If $|t_{calculated}| > t_{critic}$, The difference between the means of the levels is significant.

3.3.8 Determination of Collision Gas Pressure Range

The final instrumental operation parameter was collision gas pressure (CGP). The values for the other factor were adjusted to the values determined in the previous sections. ANOVA analysis resulted in that this factor has got a significant effect on integration area of all hormones including EE2 (Table 3.36). However, there are significant differences in areas obtained at different levels of collision gas pressure. For example, area obtained for E1 was A= 20000 at CGP= 1 arb and increased to A= 44000 at CGP= 1.5 arb and remained almost constant when CGP was 2 arb (See appendix Table 18). Statistical analysis of these results showed that the difference between CGP= 1 arb and CGP= 1.5 arb is significant but there is no significant difference between CGP= 1.5 arb and CGP= 2 arb (Figure 3.37). The situation was the same for EE2 (Figure 3.37). However, the most significant differences for E2 and E3 were observed at CGP= 1.5 arb and CGP= 2 arb. The highest integration area was obtained at CGP= 2 for these hormones (Figure 3.38 and Table 3.37). Therefore,

range for CGP value can be selected as 1.5 arb and 2 arb for optimization of these parameters in the further experiments.

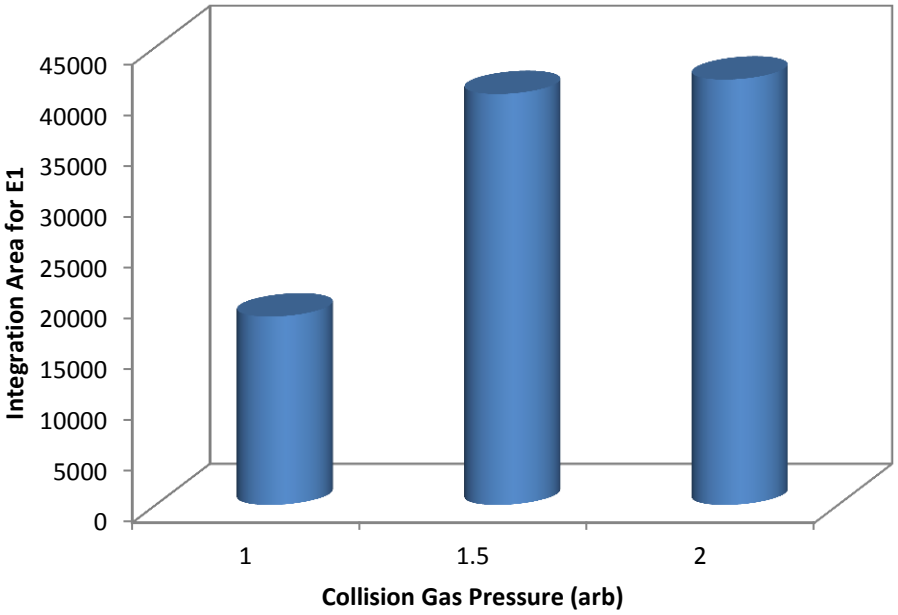
Table 3.36 ANOVA result for the significance of collision gas pressure on integration area of hormones in LC-MS/MS.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F critic*	Result
E1	1364387505	2	682193752.6	285.9900283	7.167E-09	4.2564947	Significant
E2	69260191.89	2	34630095.94	16.8914011	0.0008982	4.2564947	Significant
E3	159126244.6	2	79563122.32	34.72619049	5.866E-05	4.2564947	Significant
EE2	7920604.597	2	3960302.298	5.500366381	0.0275033	4.2564947	Significant

Table 3.37 LSD test results for significance of collision gas pressure levels on integration area of hormones.

Type of hormone	Levels	$(\overline{Y}_i - \overline{Y}_j)$ $t_{calculated}$	t_{critic} $(\alpha\ .05)$	$t_{calulated} > t_{critic}$ *
E1	1 vs 1.5	-21877	2470	Significant
	1 vs 2	-23295	2470	Significant
	1.5vs 2	-1418	2470	Insignificant
E2	1.5 vs 1	3789	2290	Significant
	1.5 vs 2	-2005	2290	Insignificant
	1 vs 2	-5794	2290	Significant
E3	1.5 vs 1	7266	2421	Significant
	1.5 vs 2	-848	2421	Insignificant
	1 vs 2	-8114	2421	Significant
EE2	1.5 vs 1	1.5 vs 1	1916	Significant
	1.5 vs 2	1.5 vs 2	493	Insignificant
	1 vs 2	1 vs 2	-1423	Significant

*If $|t_{calculated}| > t_{critic}$, The difference between the means of the levels is significant.



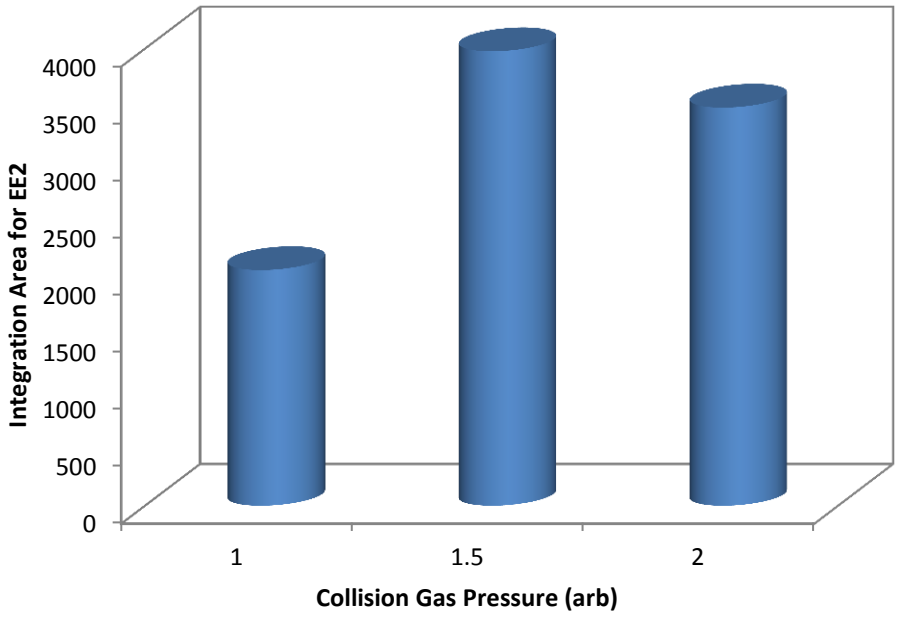
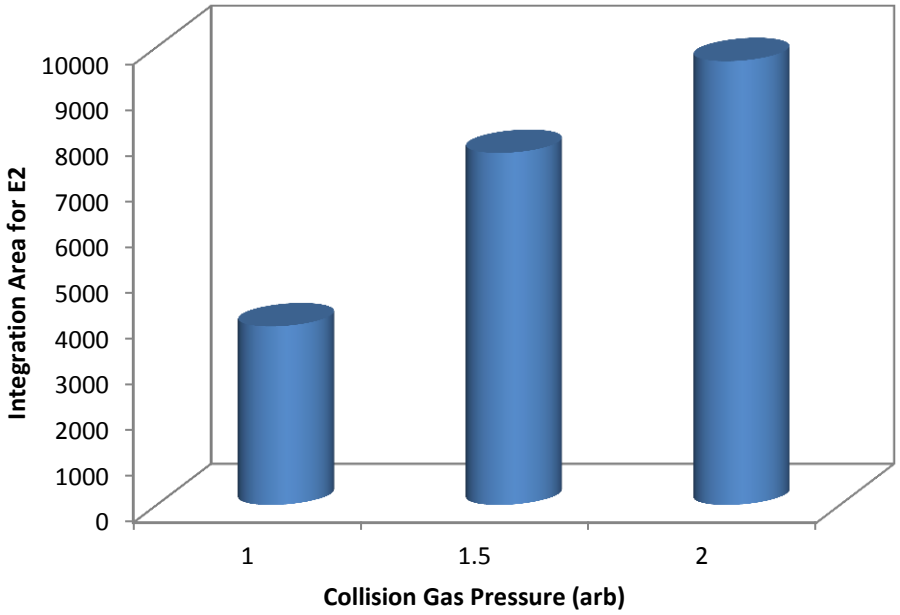


Figure 3.37 Variation of integration area of E1and EE2 with collision gas pressure.



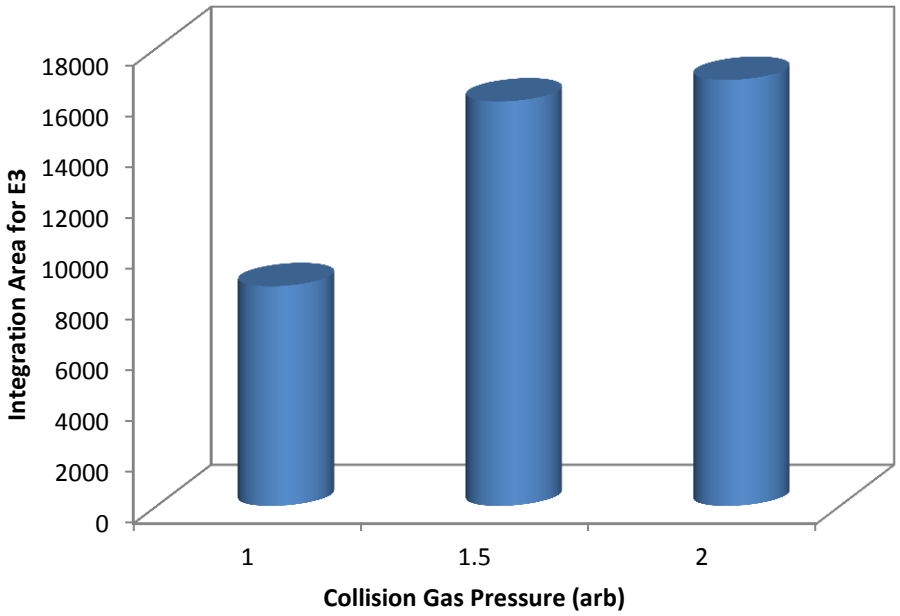


Figure 3.38 Variation of integration area of E2 and E3 with collision gas pressure.

Table 3.38 summarizes the instrument operation conditions which significantly affect the integration area of hormones. As seen from table, the significant factors are sheath gas pressure, spray voltage, vaporizer temperature, cone position and collision

gas pressure. On the other hand, capillary temperature, ion sweep gas pressure and aux gas pressure do not significantly affect the response area (Table 3.39).

Table 3.38 The values of significant LC-MS/MS operation factor to obtain high integration area of hormones.

Hormones	Sheath Gas Pressures (arb)	Spray Voltage (V)	Vaporizer Temperature (^{o}C)	Cone Position Range Max	Collision Gas Pressure Max
E1	35	2750	375	D	2
E2	35	2750	375	D	2
E3	35	2750	375	D	2
EE2	Not effect between 30-40	No effect between 2250-2750	375	D	2

Table 3.39 The insignificant LC-MS/MS operation factor for integration area of hormones.

Hormones	Capillary Temperature ^{o}C	Ion Sweep Gas (arb)	Aux Gas Pressure, (arb)
E1	No effect 300-350	No effect 0-1	No effect 10-20
E2	300	No effect 0-1	No effect 10-20
E3	No effect 300-350	No effect 0-1	No effect 10-20
EE2	No effect 300-350	No effect 0-1	No effect 10-20

3.4 Improvement of Optimization Conditions for LC Elution: Composition of Mobile Phase, Standard Solution and Flow Rate

Optimizations of these parameters were already done in the beginning of the study. However, the operation conditions of the instrument were selected arbitrarily. Since, significant and insignificant parameters were selected and the conditions to obtain the highest integration area were determined in section 3.3, the optimization was repeated in this stage of thesis. The aim of this section was to optimize flow rate through the column, composition of mobile phase and standard solution for the best peak symmetry and resolution factor in LC-MS/MS with regard to the best instrumental conditions.

Box-Behnken experimental design method was used. The Box-Behnken design is an independent quadratic design in that it does not contain an embedded factorial or

fractional factorial design. The treatment combinations are at the midpoints of edges of the process space and at the center. These designs are rotatable (or near rotatable) and require 3 levels of each factor. The designs have limited capability for orthogonal blocking compared to the central composite designs. The three independent variables in this study were % ACN in standard solution (1000 ng/L) (X_1), % ACN concentration in mobile phase (X_2), and flow rate (X_3). The ranges for the factors were determined based on the results of first optimization study. % ACN in standard solution (ACN_s) X_1= 22%-28%, % ACN in mobile phase (ACN_m) X_2 = 44%-50% and flow rate X_3= 100 – 200 µL/min. The dependent variables were peak symmetry (PS) and resolution factor (RF). Table 3.40 summarizes the coded and actual experimental points of Box-Behnken experimental design. Center point was repeated 5 times.

The experiments were conducted at isocratic conditions, no gradient was applied. The parent and product masses, LC-MS/MS running conditions and selected reaction mode (SRM) conditions were given in Table 3.41. The interface conditions given in Table 3.42 represent the conditions at which the highest integration areas of the hormones were obtained in section 3.3.

Table 3.40 The coded and experimental points of Box-Behnken design.

Run Number	Coded Variables			Actual Variables		
STD	X1	X2	X3	Standard Solution %ACN	Mobile Phase % ACN	Flow Rate µL/min
1	+1	-1	0	28.00	44.00	150.00
2	0	-1	-1	25.00	44.00	100.00
3	-1	-1	0	22.00	44.00	150.00
4	0	-1	+1	25.00	44.00	200.00
5	+1	0	+1	28.00	47.00	200.00
6	-1	0	+1	22.00	47.00	200.00
7	-1	0	-1	22.00	47.00	100.00
8	+1	0	-1	28.00	47.00	100.00
9	0	+1	0	22.00	50.00	150.00
10	0	+1	+1	25.00	50.00	200.00
11	+1	+1	0	28.00	50.00	150.00
12	0	+1	-1	25.00	50.00	100.00

Table 3.40 The coded and experimental points of Box-Behnken design (continued).

C-1	0	0	0	25.00	47.00	150.00
C -2	0	0	0	25.00	47.00	150.00
C -3	0	0	0	25.00	47.00	150.00
C -4	0	0	0	25.00	47.00	150.00
C -5	0	0	0	25.00	47.00	150.00

Table 3.41 The SRM conditions in LC-MS/MS for measurement of hormones.

	Parent Mass	Product Mass	SRM Collision Energy	Retention Time	Time Window	Tube Lens	Polarity	Trigger	Name
1	269.1	143.2	51	7.50	15.00	85	-	0	E1
2	269.1	145.3	36	7.50	15.00	85	-	0	E1
3	271.1	145.1	56	7.50	15.00	85	-	0	E2
4	271.1	183.2	44	7.50	15.00	85	-	0	E2
5	287.0	145.0	48	7.50	15.00	85	-	0	E3
6	287.0	171.1	41	7.50	15.00	85	-	0	E3
7	295.1	145.0	49	7.50	15.00	85	-	0	EE2
8	295.1	159.1	44	7.50	15.00	85	-	0	EE2

Table 3.42 Interface conditions in LC-MS/MS for optimization of composition of mobile phase, standard solution and flow rate.

Capillary Temperature (^{o}C)	300
Vaporizer Temperature (^{o}C)	375
Sheath Gas Pressure (Arb)	35
Aux Gas Pressure (Arb)	20
Ion Sweep Gas Pressure (Arb)	0
Spray Voltage (V) (positive/negative polarity)	3000/2750

The experiments were conducted randomly. Responses obtained at these experimental conditions were presented at Table 3.43. The peak symmetry obtained for E1 varied between PS= 1.58 and 2.71 after improvement of instrument running conditions. However this range was considerably wider as PS= 0.5 to 3.8 obtained before this improvement. This improvement is also valid for PS values of all hormones. For example, PS value for E3 reached to 4.652 which indicate that peak is

not symmetric. But improvement in operating conditions and addition of NH_4OH decreased maximum PS of E3 to around PS=2.0 which is acceptable.

Similarly, a substantial improvement in resolution factor was observed. The desired condition for RF is to get high value which indicate that there is a good separation of substances and hence a good chromatogram. The highest RF for E3 was RF= 2.8 before instrument operating condition was improved. That improvement provided a RF value of E3= 4.5 which means an acceptable separation between E2 and E3. Similar increases in the RF values of peak pairs of other hormones were observed. These results indicated that determination of best instrumental conditions and addition of NH_4OH into mobile phase provided significantly better chromatograms.

Table 3.43 Observed peak symmetry and resolution factors of Box-Behnken experimental design.

Run Number	Standard Solution %ACN	Mobile Phase % ACN	Flow Rate µL/min	RF-E3	RF-E2	RF-EE2	PS-E1	PS-E2	PS-E3	PS-EE2
1	22	44	150	3.95	1.09	0.39	2.24	2.00	2.10	2.00
2	28	44	150	3.73	1.29	0.59	1.91	1.41	1.79	2.00
3	22	50	150	2.70	0.62	0.48	2.41	3.00	1.62	1.61
4	28	50	150	2.05	0.57	0.54	1.78	2.38	2.25	1.75
5	22	47	100	4.05	1.08	0.59	1.82	1.65	1.64	1.36
6	28	47	100	3.07	0.93	0.45	2.32	1.69	2.46	1.42
7	22	47	200	2.50	0.74	0.51	2.71	1.75	2.00	2.33
8	28	47	200	3.10	0.78	0.39	2.22	1.92	1.60	2.06
9	25	44	100	4.50	1.25	0.75	1.86	1.86	2.00	2.50
10	25	50	100	3.05	0.79	0.65	1.58	1.19	1.44	2.00
11	25	44	200	2.90	0.91	0.49	2.14	2.62	2.36	2.67
12	25	50	200	2.11	0.47	0.53	1.79	2.70	1.06	2.00
C1	25	47	150	3.93	0.88	0.46	1.64	1.85	1.74	1.80
C2	25	47	150	3.70	1.03	0.57	2.59	1.71	1.82	1.93
C3	25	47	150	3.21	0.72	0.54	1.92	1.94	1.86	1.32
C4	25	47	150	3.47	1.08	0.52	1.92	1.94	1.57	1.69
C5	25	47	150	3.33	0.89	0.63	2.21	1.59	1.73	1.68

3.4.1 Optimization of Peak Symmetry

ANOVA analysis for the significance of coefficients of response equation for peak symmetry (Table 3.44 – Table 3.45, respectively) was conducted. Values of "Prob > F" less than 0.0500 indicate model terms are significant. Values greater than 0.1000 indicate the model terms are not significant. Significance of quadratic coefficients indicates that response equation model is quadratic (as given in equation 3.7). Insignificant quadratic coefficients state that linear response equation can be used for diagnosis of model. The lack of fit test is desired to be insignificant which indicates reproducibility of the results. Reponses equation coefficients for different hormones were given in Table 3.45 R^2 >0.90 depicts a good agreement between predicted values by using response equation coefficients and observed value. R^2 value was higher than 0.90 for E2, E3and EE2. Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable and states that model equation can be used to predict the reponse for any value of factors within tha range of experimental design. Although R^2 value for E1 vas less than 0.90, Adeq Precison is larger than 4. Therefore, model coefficient can be used to predict response for E1. The coefficients were used to predicted the reposes for the experimental points. As seen from the tables, the difference between observed and predicted values is very low or at acceptable level (Table 3.46). This result proves that model coefficients are reliable to predict response at any experimental point within the studied ranges of factors.

$$Y= b_0 + b_1X_1 + b_2X_2 + b_3X_3+b_{12}X_1X_2 + b_{13} X_1X_3+b_{23}X_2X_3+b_{123}X_1X_2X_3+b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 \quad (3.7)$$

Table 3.44 ANOVA analysis of peak symmetry of hormones for improved instrumental running conditions.

Source of variation		**E1**	**E2**	**E3**	**EE2**
Coded	**Actual**	**p-value Prob > F**	**p-value Prob > F**	**p-value Prob > F**	**p-value Prob > F**
M	Model	0.6747	0.0614	0.0030	0.0629
X_1	% ACN in standard	0.3890	0.2411	0.0480	0.9115
X_2	% ACN in mobile phase	0.4098	0.3137	0.0003	0.0395

Table 3.44 ANOVA analysis of peak symmetry of hormones for improved instrumental running conditions (continued).

X_3	Flow rate	0.5246	0.0077	0.9440	0.7089
X_1X_2	Interatction between mobile phase and stationary phase	0.6893	0.9608	0.0058	0.7552
X_1X_3	% ACN in mobile phase vs flow rate	0.2265	0.8134	0.0019	0.4654
X_2X_3	% ACN in stationary phase vs flow rate	0.9244	0.2090	0.0148	0.7089
X_1^2	Quadratic effect of mobile phase	0.2477	0.8396	0.0092	0.1557
X_2^2	Quadratic effect of stationary phase	0.3067	0.0356	0.8908	0.0245
X_3^2	Quadratic effect of flowrate	0.9363	0.5624	0.7155	0.0416
$X_1^2X_2$		0.5292	0.0183	0.0014	0.4166
X_12X_3		0.7776	0.0484	0.1499	0.0589
Lack of Fit		0.3928	0.0305	0.7970	0.6179

Table 3.45 Regression coefficients of peak symmetry for estrogenic hormones.

Coefficient of response equation	**Coefficients for E1**	**Coefficients for E2**	**Coefficients for E3**	**Coefficients for EE2**
b_0	-246.26	-492.878	-441.752	-141.667
b_1	15.46322	47.50023	36.10214	18.38937
b_2	6.201013	10.7824	10.13496	-0.5126
b_3	0.155584	-0.71428	-0.05877	0.498594
b_{12}	-0.32482	-1.18659	-0.82847	-0.24048
b_{13}	-0.01002	0.053843	0.011562	-0.04091
b_{23}	-0.00012	0.001263	-0.00124	-0.00028
b_{11}	-0.29722	-0.95077	-0.73982	-0.36981
b_{22}	-0.02204	0.040647	-0.0008	0.036328
b_{33}	-5.9E-06	-3.2E-05	-7.7E-06	0.000112
b_{11} b_2	0.006328	0.023717	0.017091	0.004887
$b_{11}b_3$	0.000168	-0.00107	-0.00027	0.000807
R^2	0.625287	0.903189	0.973602	0.902112
Adequate precision	4.176596	7.391553	16.27569	7.638518

Table 3.46 Actual and predicted values of peak symmetries of hormones.

Run No:	X1	X2	X3	E1		E2		E3		EE2	
				Actual	Predicted	Actual	Predicted	Actual	Predicted	Actual	Predicted
1	22	44	150	2.24	2.12	2.00	1.82	2.10	2.08	2.00	2.04
2	28	44	150	1.91	2.03	1.41	1.59	1.79	1.80	2.00	1.96
3	22	50	150	2.41	2.29	3.00	2.82	1.62	1.60	1.61	1.65
4	28	50	150	1.78	1.90	2.38	2.56	2.25	2.26	1.75	1.71
5	22	47	100	1.82	1.94	1.65	1.83	1.64	1.65	1.36	1.31
6	28	47	100	2.32	2.20	1.69	1.51	2.46	2.45	1.42	1.46
7	22	47	200	2.71	2.83	1.75	1.93	2.00	2.01	2.33	2.29
8	28	47	200	2.22	2.10	1.92	1.75	1.60	1.59	2.06	2.11
9	25	44	100	1.86	1.86	1.86	1.86	2.00	2.00	2.50	2.50
10	25	50	100	1.58	1.58	1.19	1.19	1.44	1.44	2.00	2.00
11	25	44	200	2.14	2.14	2.62	2.62	2.36	2.36	2.67	2.67
12	25	50	200	1.79	1.79	2.70	2.70	1.06	1.06	2.00	2.00
C1	25	47	150	1.64	2.06	1.85	1.81	1.74	1.74	1.80	1.68
C2	25	47	150	2.59	2.06	1.71	1.81	1.82	1.74	1.93	1.68
C3	25	47	150	1.92	2.06	1.94	1.81	1.86	1.74	1.32	1.68
C4	25	47	150	1.92	2.06	1.94	1.81	1.57	1.74	1.69	1.68
C5	25	47	150	2.21	2.06	1.59	1.81	1.73	1.74	1.68	1.68

Table 3.47 Optimum predicted conditions for the best peak symmetry.

%ACNs	%ACN_m	Flow rate	PS E1	PS E2	PS E3	PS EE2	Desirability
25	50	105	1,60	1,27	1,46	1,95	1
25	50	100	1,60	1,18	1,46	1,99	1
24	50	100	1,59	1,26	1,35	1,95	1
24	50	100	1,60	1,36	1,32	1,92	1
25	50	103	1,59	1,24	1,42	1,96	1
25	50	105	1,60	1,27	1,46	1,95	1

Two level interactions of the factors for different hormones at constant ACN_s concentration of 25%, which was determine according to results of optimization, were evaluated (Table 3.47). Figure 3.39 depicts the variation of PS value of E1 at different %ACN_m and flow rates. Increasing flow rate adversely affects the peak symmetry. It was PS= 1.57 at 100 µL/min flow rate and increased to PS= 2.10 when flow rate was F= 200 µL/min at ACN_m = 44%. The PS value was higher for % ACN_m=44% - 47% at any flow rate, but showed a substantial decrease at

ACN_m>47%. The minimum PS value was obtained as PS= 1.5 at ACN_m= 50 % and flow rate = 100 µL/min.

Variation of PS of E2 at different flow rate and %ACN_m concentration is shown in Figure 3.40. The lowest PS value around PS= 1.0 was observed at ACN_m>48% and flow rate= 100 µL/min. The effect of flow rate was adverse on the PS and more significant than that of ACN_m on PS for E2. Increasing flow rate from 100 µL/min to 200 µL/min at 50% ACN_m resulted in increasing PS value from PS= 1.1 to PS= 2.3. However, increasing ACN_m concentration provided better PS values. It was PS= 1.4 at 44% ACN_m but decreased to around PS= 1.0 at 50% ACN_m (flow rate = 100 µL/min).

The most significant factor in the case of PS value of E3 was ACN_m concentration. As seen from Figure 3.41 increasing ACN_m from 44% to 50% resulted in a substantial improvement peak symmetry with decreasing in the value from PS= 2.3 to PS=1.1 at flow rate= 100 µL/min. The effect of flow rate on PS was not as strong as ACN_m. Decrease in PS from PS= 1.4 to PS= 1.2 when flow rate was increased from 100 µL/min to 200 µL/min at ACN_m= 50%. The best peak symmetry around PS= 1 for E3 can be obtained at flow rate= 200µL/min and ACN_m= 50%.

Finally, variation of PS for EE2 with ACN_m and flow rate at ACN_s= 25 % is depicted in Figure 3.42 the main factor that affects the PS value of EE2 is ACN_m rather than flow rate. PS value most significantly increases from PS= 1.9 to PS= 2.5 when ACN_m was decreased from 50% to 44% at minimum flow rate of 100 µL/min. However, PS value varies between 1.8 and 1.9 for flow rate between 100 µL/min and 200 µL/min at ACN_m= 50%. This slight variation in PS value for different values of flow rate can be observed even at lowest concentration of ACN_m= 44%. It was 2.7 at 200 µL/min and decreased to only 2.4 at 100 µL/min flow rate. In summary the best acceptable peak symmetry for EE2 can be obtained at flow rate= 100 µL/min, ACN_m= 50%.

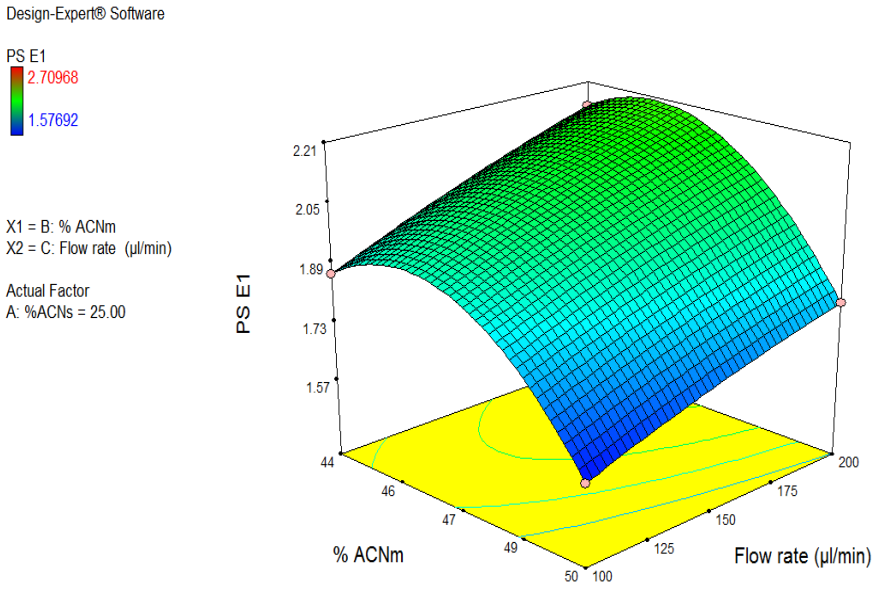


Figure 3.39 Variation of peak symmetry of E1 with % ACN_m and flow rate at ACN_s= 25%.

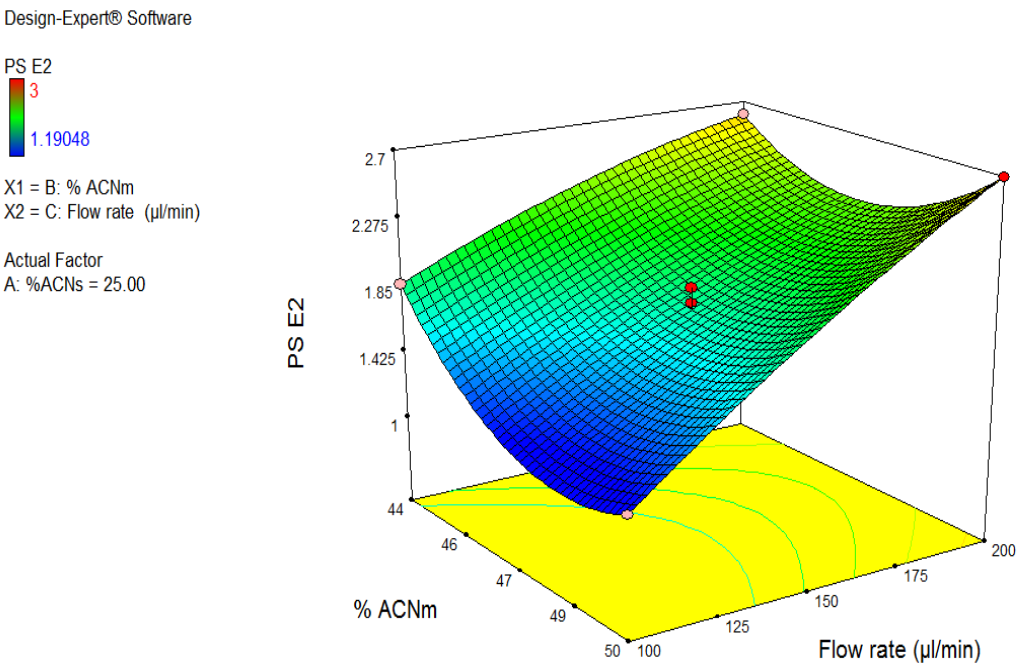


Figure 3.40 Variation of peak symmetry of E2 with % ACN_m and flow rate at ACN_s= 25%.

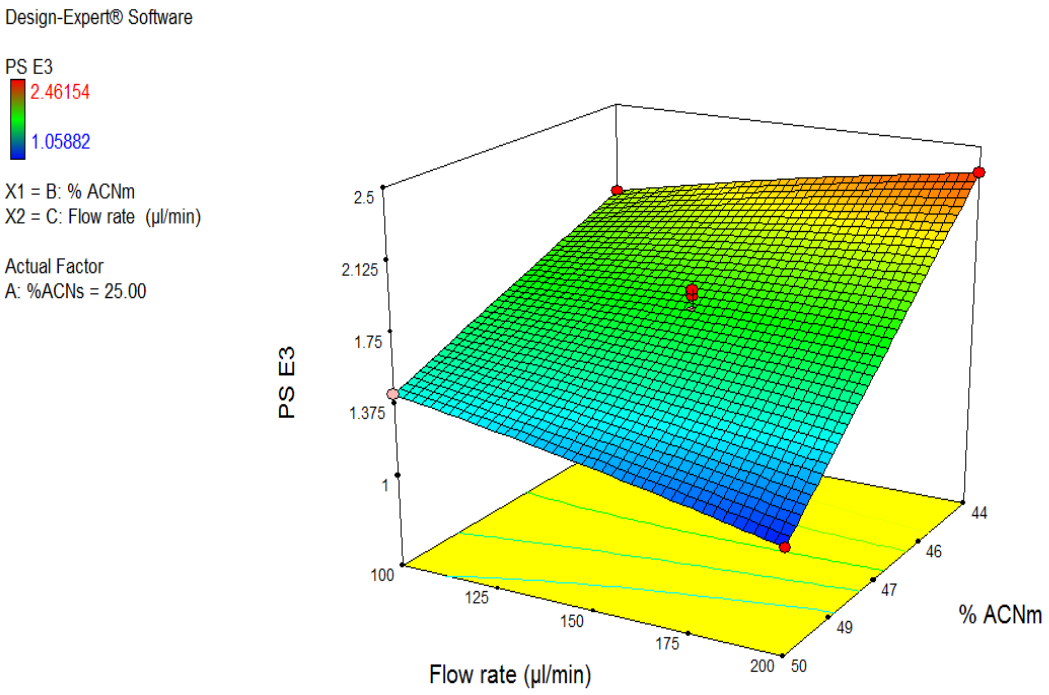


Figure 3.41 Variation of peak symmetry of E3 with % ACN_m and flow rate at ACN_s= 25%.

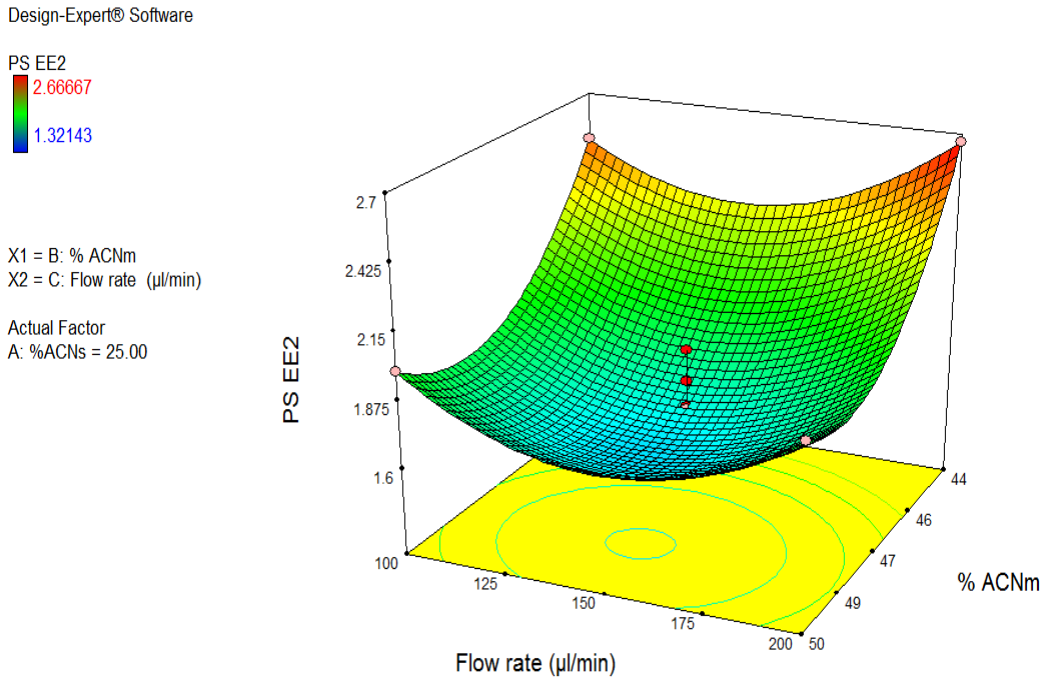


Figure 3.42 Variation of peak symmetry of EE2 with % ACN_m and flow rate at ACN_s= 25%.

3.4.2 Optimization of Resolution Factor

Variance analysis of Box-Behnken design for resolution factor response is given in Table 3.48 similar to peak symmetry, variance analysis for the significance of coefficients of resolution factor response equation (Table 3.49) was carried out. Response equation for E2 and EE2 were quadratic but it was linear for E3. Therefore no significance of the quadratic terms of this hormone was conducted. ANOVA results indicated that the most significant linear effect corresponds to the flow rate for all hormones. The lack of fit for all hormones was insignificant which indicates reproducibility of the results. Response equation coefficients for different hormones were given in Table 3.49. Regression coefficients between observed and predicted values were higher than 0.85 for E3 and E2 but it was 0.57 for EE2. However, Adeq Precision for this hormone was greater than 4 which means that model equation can be used to predict the reponse for any value of factors within tha range of experimental design. Moreover, the difference between observed and predicted values were very low or at acceptable level too (Table 3.50). This result prooves that model coefficnets are reliable to predict response at any experimental point within the studied ranges of factors.

Optimization of condition by using response equation of resolution factor was done. The high resolution factor indicates that there is a good separation of peaks relative to each other. Therefore, the main aim in the optimization of RF was to select the conditions to obtain the highest RF values for each hormone. In other words, the target in the optimization was to maximization of RF value. Table 3.51 depicts the optimum conditions with 95% guarantee target aim. As seen from table, $\%ACN_s$= 25, $\% ACN_m$= 44 and flow rate= 100 μL/min are the optimal conditions to get the best RF values.

Table 3.48 ANOVA analysis of resolution factor of hormones for improved instrumental running conditions.

Source of variation		**E2**	**E3**	**EE2**
Coded	**Actual**	**p-value Prob > F**	**p-value Prob > F**	**p-value Prob > F**
M	Model	0.0006	0.0035	0.4857
X_1	% ACN in standard	0.9010	*0.1632*	0.9853
X_2	% ACN in mobile phase	< 0.0001	*0.0003*	0.9493
X_3	Flow rate	0.0031	*0.0014*	0.0812
X_1X_2	Interatction between mobile phase and Stationary phase	0.2733	*0.4713*	0.4978
X_1X_3	%ACN in mobile phase vs flowrate	0.3835	*0.0265*	0.8815
X_2X_3	%ACN in stationary phase vs flowrate	0.9221	*0.2779*	0.4793
X_1^2	Quadratic effect of mobile phase	Not included to response equation	*0.2105*	0.1073
X_2^2	Quadratic effect of stationary phase	Not included to response equation	*0.1358*	0.4182
X_3^2	Quadratic effect of flowrate	Not included to response equation	*0.2877*	0.6229
Lack of Fit		0.9308	*0.5253*	0.1296

Values of "Prob > F" less than 0.0500 indicate model terms are significant. Values greater than 0.1000 indicate the model terms are not significant.

Table 3.49 Regression coefficients of resolution factor response equation for estrogenic hormones.

Coefficient of response equation	**Coefficients for E2**	**Coefficients for E3**	**Coefficients for EE2**
b_0	-1.07978	-51.3902	2.170366
b_1	0.26869	1.167873	0.616001
b_2	0.075353	2.339036	-0.34164
b_3	-0.01237	-0.10904	-0.01582
b_{12}	-0.00669	-0.01194	-0.0036
b_{13}	0.000316	0.002635	4.67E-05
b_{23}	3.47E-05	0.001107	0.000226

Table 3.49 Regression coefficients of resolution factor response equation for estrogenic hormones (continued).

b_{11}		-0.02107	-0.00907
b_{22}		-0.02577	0.004225
b_{33}		-6.3E-05	9.09E-06
R^2	0.8708	0.9253	0.5744
Adequate precision	12.079	10.648	4.224

Table 3.50 Actual and predicted values of resolution factor for hormones.

Run				E3		E2		EE2	
Number	X1	X2	X3	Actual	Predicted	Actual	Predicted	Actual	Predicted
1	22	44	150	3.95	3.80	1.09	1.08	0.39	0.47
2	28	44	150	3.73	3.70	1.29	1.21	0.59	0.53
3	22	50	150	2.70	2.72	0.62	0.69	0.48	0.53
4	28	50	150	2.05	2.20	0.57	0.57	0.54	0.47
5	22	47	100	4.05	4.24	1.08	1.07	0.59	0.56
6	28	47	100	3.07	3.14	0.93	0.99	0.45	0.54
7	22	47	200	2.50	2.43	0.74	0.70	0.51	0.41
8	28	47	200	3.10	2.91	0.78	0.80	0.39	0.43
9	25	44	100	4.50	4.46	1.25	1.30	0.75	0.71
10	25	50	100	3.05	2.83	0.79	0.77	0.65	0.63
11	25	44	200	2.90	3.11	0.91	1.00	0.49	0.51
12	25	50	200	2.11	2.15	0.47	0.49	0.53	0.57
C1	25	47	150	3.93	3.53	0.88	0.89	0.46	0.54
C2	25	47	150	3.70	3.53	1.03	0.89	0.57	0.54
C3	25	47	150	3.21	3.53	0.72	0.89	0.54	0.54
C4	25	47	150	3.47	3.53	1.08	0.89	0.52	0.54
C5	25	47	150	3.33	3.53	0.89	0.89	0.63	0.54

Table 3.51 Optimum predicted conditions to obtain the maximum resolution factor values.

X1	X2	X3	E3	E2	EE2	Desirability
24.69	44	100	4.50	1.30	0.70	0.95
24.74	44	100	4.49	1.30	0.70	0.95
24.98	44	100	4.46	1.30	0.71	0.95
24.63	44	101.02	4.50	1.29	0.70	0.95

Variation of RF values with two level interactions of factors was evaluated. % ACN_s was kept constant at 25% which is the optimal concentration obtained after

optimization. Figure 3.43 depicts effect of % ACN_m and flow rate on RF value of E3. Both factors significantly affect the RF value. For example, decreasing flow rate from 200 µL/min to 100 µL/min at 50 % ACN_m resulted in increasing in RF value from RF=2.1 to RF= 2.8. Similarly, there was an increase from 2.1 to around 3.2 for ACN_m concentration 50% to 44%, respectively. However, the interaction effects of these factors are more significant than the main factor effects. Since, decreasing both factors provide a substantial increase in the RF value. The maximum resolution factor value with regard to E3 can be obtained as RF=4.5 at the low levels of factor as ACN_m= 44% and flow rate= 100 µL/min.

The relationship between factors for E2 was linear and curvation was insignificant (Table 3.48). Similar to RF value of E3, the combined effect of factors are more significant than the main effects of factors (Figure 3.44). As a results, maximum RF value can be obtained as RF= 1.3 at ACN_m= 44% and flow rate= 100 µL/min. In the case of EE2, the main increase in the response can be obtained with the decrease in flow rate from 200 µL/min to 100 µL/min (Figure 3.45). The interaction effects of these two factors provide a substantial improvement in the response as well.

Although statistically proved that the model equations can be used to predict the responses at any values of factor within the studied range, the best approach is to run an experiment at the point which is different than design points. In other words, model verification was conducted to be sure about the model. Table 3.52 depicts the observed and predicted values of two different values of investigated factors. The results indicated that there is no substantial difference between observed and predicted values. The chromatograms of these two conditions were given in Figure 3.46 and Figure 3.47.

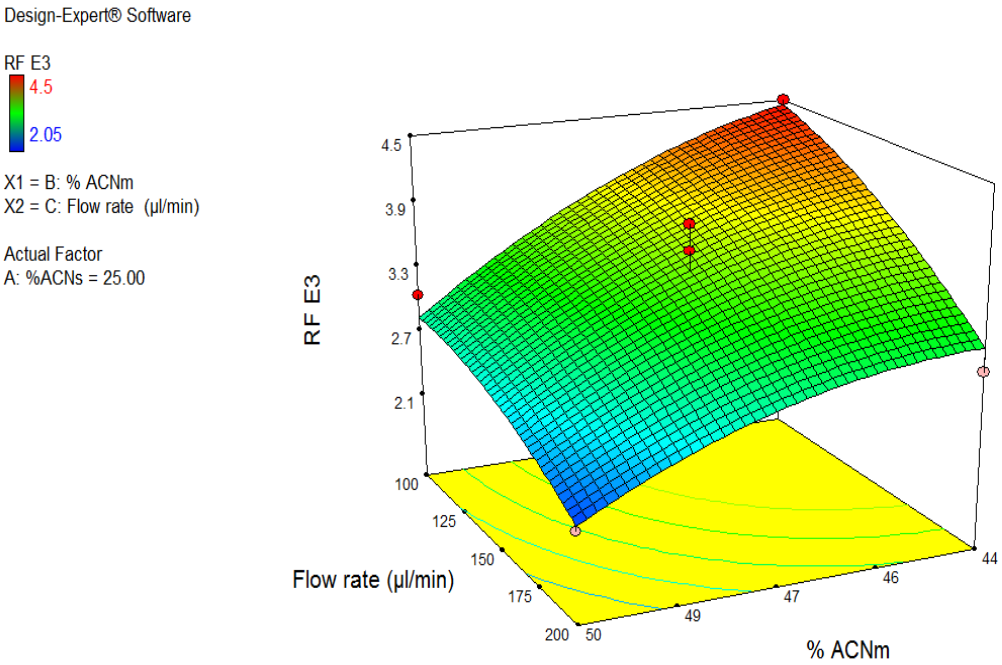


Figure 3.43 Variation of RF value of E3 with % ACN_m and flow rate at ACN_s= 25%.

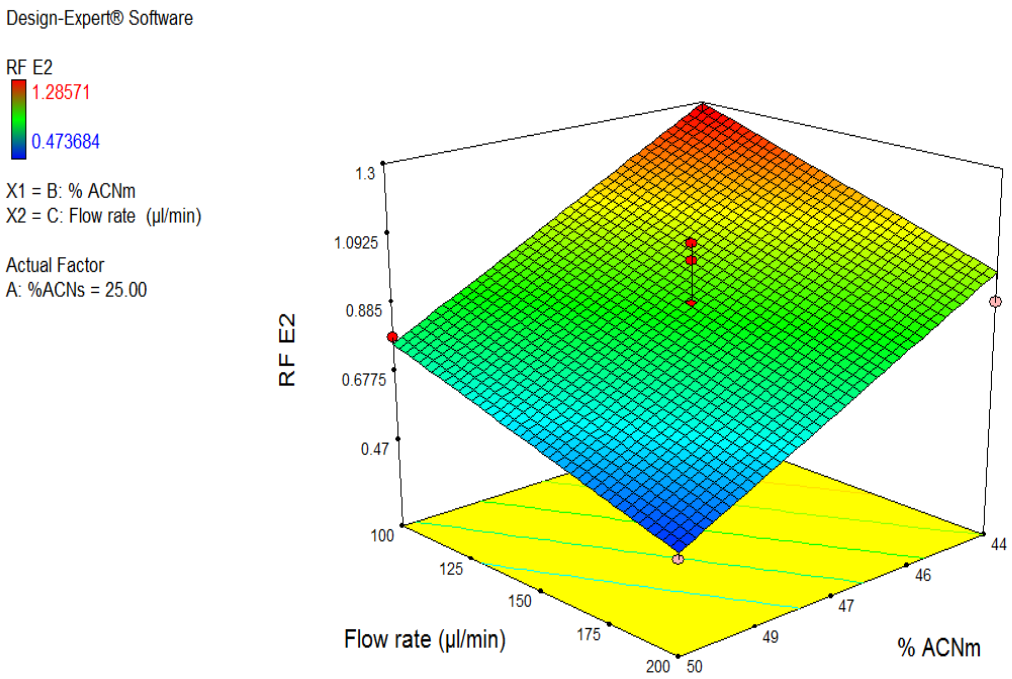


Figure 3.44 Variation of RF value of E2 with % ACN_m and flow rate at ACN_s= 22%.

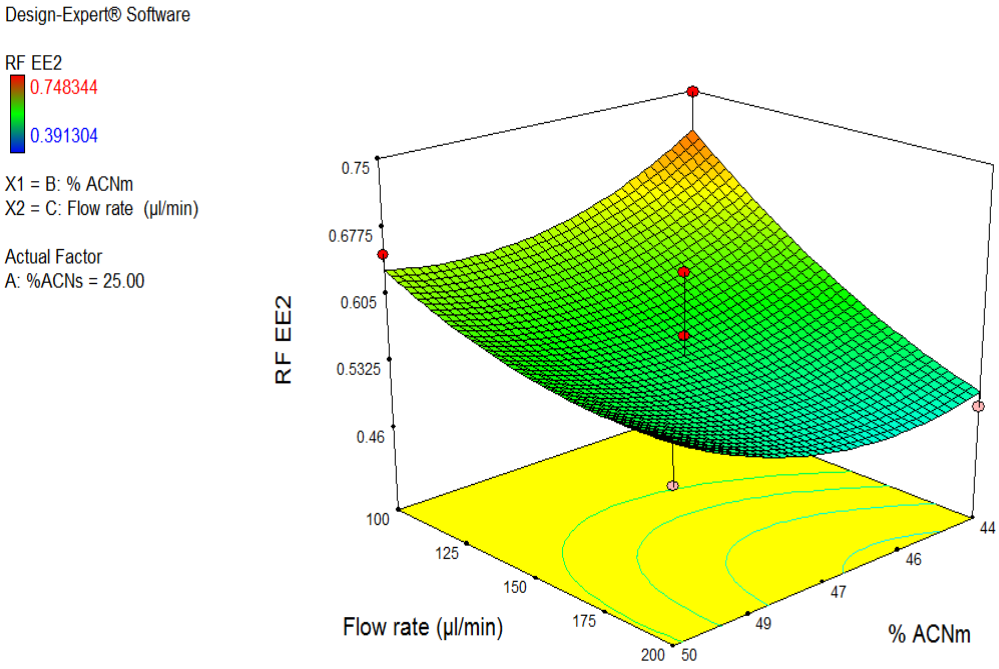


Figure 3.45 Variation of RF value of EE2 with % ACN_m and flow rate at ACN_s= 25%.

Table 3.52 Observed and predicted values of RF and PS at the experimental points which are different than design points.

Response	**%ACN_s**	**%ACN_m**	**Flow**	**%ACN_s**	**%ACN_m**	**Flow**
	25	**48**	**145**	**25**	**44**	**175**
	Observed	**Predicted**		**Observed**	**Predicted**	
RF E3	3.16	3.33		3.05	3.57	
RF E2	0.717	0.817		1.00	1.08	
RF EE2	0.521	0.552		0.588	0.540	
PS E1	1.94	1.97		2.06	2.084	
PS E2	1.85	1.73		1.93	2.49	
PS E3	1.62	1.59		2.67	2.29	
PS EE2	1.45	1.62		2.36	2.41	

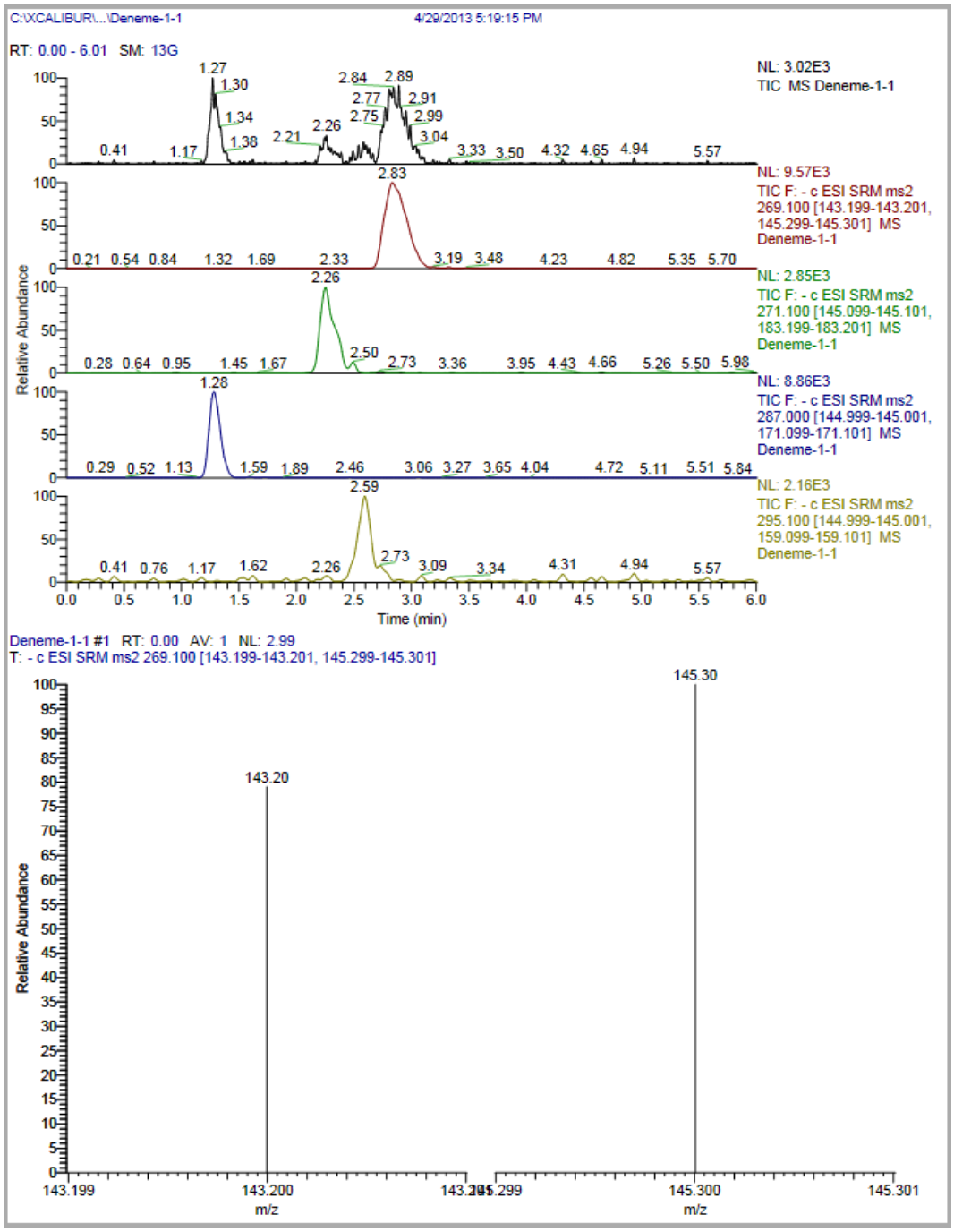


Figure 3.46 Chromatograms of hormones at ACN_s= 25%, ACN_m= 48% and flow rate = 145 µL/min.

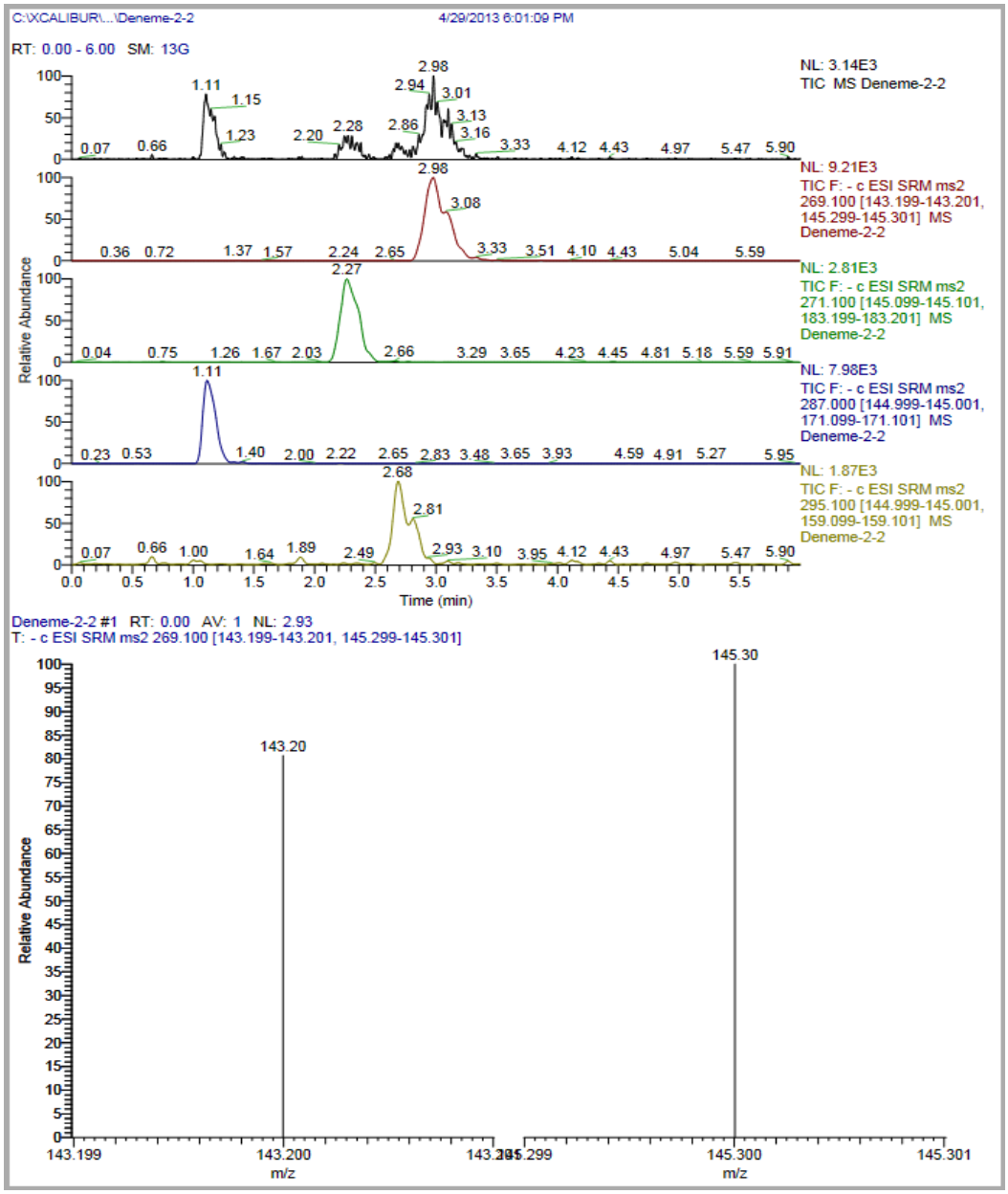


Figure 3.47Chromatograms of hormones at ACN_s= 25%, ACN_m= 44% and flow rate = 175 µL/min.

The optimization of conditions was carried out for two different responses so far in order to observe that if there are considerable differences for the optimized conditions. Fortunately, these two conditions were not substantially different. Since, LC-MS/MS cannot be operated at different conditions to obtain the best RF and PS values. Therefore, the optimization process were run again to get the maximum RF value and target PS value between PS=1 and PS=2. The results were given in Table 3.53 indicated the conditions were very close to the values obtained from individual

optimization of responses. However, EE2 is the most difficult hormone to measure. In addition there is no need to have RF=4 for E3. Therefore, the conditions needed further improvement in terms of peak symmetry of EE2 and RF of E3. The second optimization (Table 3.54) resulted in a slight variations from the Table 3.53 for the responses but acceptable.

Table 3.53 Predicted optimum conditions for peak symmetry and resolution factor.

Number	$\%ACN_s$	$\%ACN_m$	Flow rate	RF E3	RF E2	RF EE2	PS E1	PS E2	PS E3	PS EE2	Desirability
1	22.49	44	100	4.69	1.28	0.63	1.68	1.90	1.77	2.00	0.869
2	22.51	44.03	100	4.69	1.28	0.63	1.69	1.89	1.77	2.00	0.869
3	22.57	44.14	100	4.68	1.27	0.63	1.71	1.87	1.77	1.99	0.866
4	22.5	44	101.09	4.68	1.28	0.62	1.69	1.90	1.78	2.00	0.864
5	22.45	44.04	100	4.69	1.28	0.62	1.68	1.89	1.76	1.97	0.864

Table 3.54 Predicted optimum conditions and determined conditions.

	$\%ACN_s$	$\%ACN_m$	Flow rate	RF E3	RF E2	RF EE2	PS E1	PS E2	PS E3	PS EE2
Determined Conditions	28	44	136.9	3.76	1.25	0.56	2.04	1.60	1.86	1.88

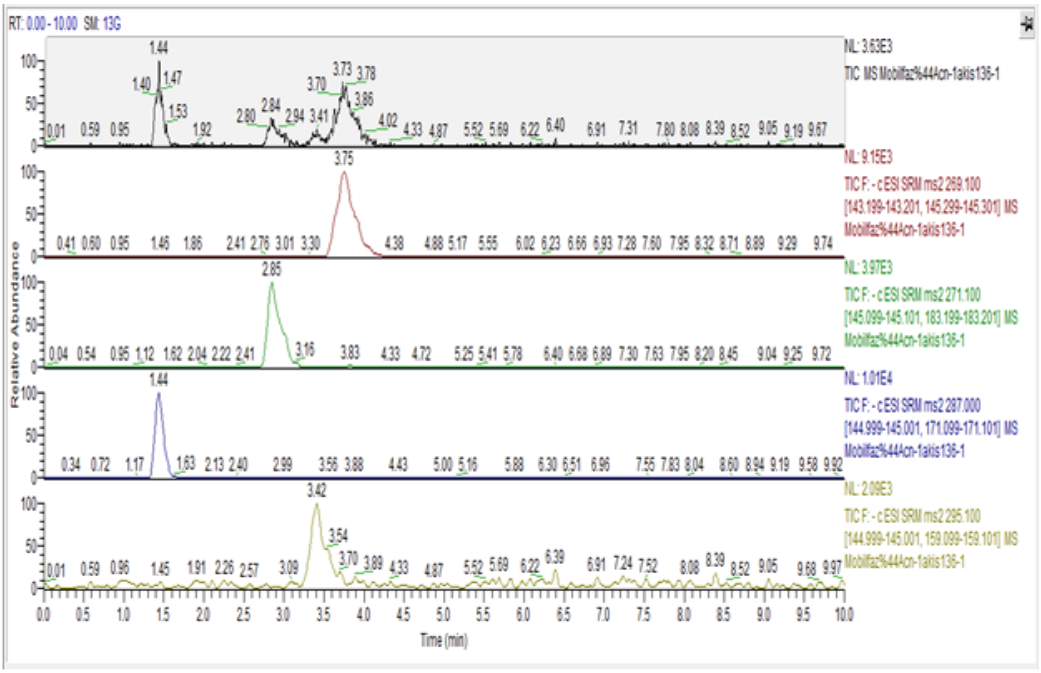


Figure 3.48 Chromatograms of hormones at ACN_s= 28%, ACN_m= 44% and flow rate = 136.9 µL/min.

3.5 Improvement of Optimized Conditions for LC-MS/MS Operation

Box-Behnken Experimental Design method was used for optimization of instrument operating conditions for the new elution conditions as determined in section 3.4. The factors were sheath gas pressure (SGP), ion sweep gas pressure (ISGP), aux gas pressure (AGP), spray voltage (SV), capillary temperature (CT), vaporizer temperature (VT) and collision gas pressure (CGP). Cone position was kept at D. The ranges of these factors were determined according to the results obtained at section 3.3 and given in Table 3.55. Table 3.56 indicates the experimental points (62). Center point repeated 6 times (run number 11, 15, 27, 40, 50, 61) times. The response was the integration area of the hormones. The observed responses for four hormones were given in Table 3.57. ANOVA analysis (Table 3.58) for the significance of coefficients (Table 3.59) of response equation for integration area of hormones was conducted. Values of "Prob > F" less than 0.0500 indicate model terms are significant. Values greater than 0.1000 indicate the model terms are not significant. The response equation included main effects, two level interactions and quadratic effects of the factors. The responses for the same experimental points were predicted by using model coefficients. The regression coefficient and Adeq Precision values were R>0.90 and AP>4, respectively, indicate that model can be used to predict the integration area of hormones for the studied ranges of factors. Finaly, optimization process was run for all hormones (1000 ng/L). The results were presented in Table 3.60. The target was to maximize the integration area. There was 14 different possible optimized conditions of the factors.

Table 3.55 Determined high levels, low levels and center points of the factors.

	Low level (-1)	High Level (+1)	Center point (0)
SGP (arb)	32	38	35
ISGP (arb)	0	2	1
AGP (arb)	10	20	15
CT (^{o}C)	250	350	300
VT (^{o}C)	325	425	375
CGP (mTorr)	1.5	2.5	2
SV (V)	2500	3000	2750

Table 3.56 Box Behnken Experimental Design for optimization of LC-MS/MS operating conditions.

Run Number	SGP	ISGP	AGP	CT	VT	CGP	SV
1	35	1	10	350	375	2	2500
2	32	2	15	350	375	2	2750
3	35	1	10	350	375	2	3000
4	35	1	15	250	325	1.5	2750
5	35	1	20	250	375	2	3000
6	35	0	20	300	375	2.5	2750
7	35	1	15	250	325	2.5	2750
8	35	0	10	300	375	2.5	2750
9	38	1	15	300	375	2.5	3000
10	32	1	15	300	375	2.5	3000
11	35	1	15	300	375	2	2750
12	35	0	15	300	425	2	3000
13	38	1	20	300	325	2	2750
14	35	1	20	350	375	2	3000
15	35	1	15	300	375	2	2750
16	35	0	20	300	375	1.5	2750
17	32	1	10	300	325	2	2750
18	32	0	15	250	375	2	2750
19	32	2	15	250	375	2	2750
20	35	1	15	250	425	2.5	2750
21	32	1	20	300	325	2	2750
22	38	2	15	250	375	2	2750
23	38	1	15	300	375	1.5	3000
24	35	0	10	300	375	1.5	2750
25	35	1	20	250	375	2	2500
26	35	1	10	250	375	2	3000
27	35	1	15	300	375	2	2750
28	35	1	15	250	425	1.5	2750
29	35	1	15	350	325	1.5	2750
30	32	1	15	300	375	2.5	2500
31	32	0	15	350	375	2	2750
32	38	1	10	300	425	2	2750
33	35	2	15	300	325	2	2500
34	32	1	20	300	425	2	2750
35	38	1	10	300	325	2	2750
36	35	1	15	350	425	1.5	2750
37	38	0	15	350	375	2	2750
38	38	1	15	300	375	1.5	2500

Table 3.56 Box Behnken Experimental Design for optimization of LC-MS/MS operating conditions (continued).

39	35	2	10	300	375	1.5	2750
40	35	1	15	300	375	2	2750
41	35	1	15	350	325	2.5	2750
42	35	1	20	350	375	2	2500
43	38	1	15	300	375	2.5	2500
44	35	2	15	300	425	2	3000
45	35	2	15	300	325	2	3000
46	38	1	20	300	425	2	2750
47	35	2	15	300	425	2	2500
48	35	2	20	300	375	2.5	2750
49	38	2	15	350	375	2	2750
50	35	1	15	300	375	2	2750
51	32	1	10	300	425	2	2750
52	32	1	15	300	375	1.5	3000
53	35	0	15	300	325	2	3000
54	32	1	15	300	375	1.5	2500
55	38	0	15	250	375	2	2750
56	35	2	10	300	375	2.5	2750
57	35	0	15	300	325	2	2500
58	35	1	10	250	375	2	2500
59	35	0	15	300	425	2	2500
60	35	2	20	300	375	1.5	2750
61	35	1	15	300	375	2	2750
62	35	1	15	350	425	2.5	2750

Table 3.57 Coefficients of model terms for different hormones.

Coefficients	E1	E2	E3	EE2
b_0	172463.6	42673.08	75588.75	26534.69
b_1	2696.783	1496.183	1969.335	370.2664
b_2	-5943.64	-3189.39	-3706	-2019.15
b_3	1313.332	-279.06	289.9176	-98.3677
b_4	-20611.1	-9877.67	-5215.27	-4739.28
b_5	-63301.3	-17206.5	-27668.8	-10228
b_6	-1278.89	-1851.56	-1267.68	-787.092
b_7	-10495	-3949.05	-5276.8	-2635.8
b_{12}	3123.884	367.206	265.28	2527.171
b_{13}	22051.21	4768.905	8073.63	1524.091
b_{14}	252.3905	386.5508	2165.158	-310.661

Table 3.57 Coefficients of model terms for different hormones (continued).

b_{15}	18513.91	4094.343	6921.138	4104.024
b_{16}	-535.769	308.2959	1576.173	526.8978
b_{17}	1921.665	-704.53	3019.263	1095.008
b_{23}	-324.297	-813.049	-2415.97	-52.0269
b_{24}	-4448.24	-616.791	-1449.26	148.2908
b_{25}	2913.445	2146.758	-722.698	1573.113
b_{26}	-1160.33	1336.474	2584.524	-578.253
b_{27}	2342.146	543.0506	1860.682	1985.744
b_{34}	2240.425	-129.756	725.704	407.2529
b_{35}	15702.7	5787.002	5086.341	2979.356
b_{36}	-3881.99	-31.0186	-297.02	-1492.05
b_{37}	-2076.36	-723.366	363.4076	495.9503
b_{45}	12118.37	5664.571	2492.89	2598.758
b_{46}	3311.824	-1.3249	63.20756	297.5233
b_{47}	8374.847	3739.379	2718.584	1212.984
b_{56}	1435.664	687.1358	1705.947	1072.538
b_{57}	-2806	1122.957	-789.546	814.9745
b_{67}	2618.567	1278.819	430.395	-779.864
b_{11}	6011.336	974.9913	3019.214	450.7009
b_{22}	-971.644	854.6614	-176.116	1199.041
b_{33}	-93.3027	309.9898	-383.987	-577.002
b_{44}	-13418	-3054.52	-5945.89	-2007.78
b_{55}	-64789.2	-16137.7	-28368.1	-10214.5
b_{66}	-12216.1	-2746.48	-5241.74	-2774.85
b_{77}	-25650.7	-6714.02	-12386.7	-5172.58

Table 3.58 Variance analysis (ANOVA) of model terms for different hormones.

	Prob > F			
Source of Variation	E1	E2	E3	EE2
Model	< 0.0001	< 0.0001	< 0.0001	< 0.0001
A	0.4618	0.2192	0.3137	0.6202
B	0.1118	0.0125	0.0641	0.0111
C	0.7190	0.8162	0.8809	0.8950
D	< 0.0001	< 0.0001	0.0115	< 0.0001
E	< 0.0001	< 0.0001	< 0.0001	< 0.0001
F	0.7260	0.1313	0.5142	0.2961
G	0.0074	0.0026	0.0106	0.0014
AB	0.6216	0.8598	0.9369	0.0588

Table 3.58 Variance analysis (ANOVA) of model terms for different hormones (continued).

AC	0.0016	0.0286	0.0222	0.2440
AD	0.9681	0.8525	0.5200	0.8099
AE	0.0065	0.0573	0.0471	0.0035
AF	0.9324	0.8821	0.6389	0.6836
AG	0.7611	0.7349	0.3715	0.3996
BC	0.9590	0.6960	0.4733	0.9679
BD	0.4832	0.7668	0.6661	0.9086
BE	0.6452	0.3065	0.8294	0.2296
BF	0.8542	0.5218	0.4433	0.6548
BG	0.7110	0.7940	0.5800	0.1325
CD	0.7230	0.9502	0.8287	0.7526
CE	0.0186	0.0092	0.1376	0.0278
CF	0.5402	0.9881	0.9294	0.2538
CG	0.7425	0.7281	0.9137	0.7012
DE	0.0636	0.0106	0.4595	0.0524
DF	0.6009	0.9995	0.9850	0.8178
DG	0.1921	0.0808	0.4203	0.3515
EF	0.8202	0.7412	0.6117	0.4092
EG	0.6574	0.5900	0.8139	0.5294
FG	0.6788	0.5398	0.8979	0.5472
A^2	0.2229	0.5437	0.2482	0.6508
B^2	0.8416	0.5942	0.9456	0.2341
C^2	0.9847	0.8464	0.8817	0.5628
D^2	0.0098	0.0649	0.0281	0.0516
E^2	< 0.0001	< 0.0001	< 0.0001	< 0.0001
F^2	0.0175	0.0949	0.0505	0.0091
G2	< 0.0001	0.0003	< 0.0001	< 0.0001
Lack of Fit	0.0212	0.0551	0.5082	0.1522

A-Sheath Gas pressure, B-Ion sweep gas pressure, C-Aux gas pressure, D-Capillary temperature, E-Vaporizer temperature, F-Collision gas pressure, G-Spray voltage.

Table 3.59 Observed and predicted integration areas of hormones at several experimental conditions.

	EE2		E2		E3		E1	
	Observed	Predicted	Observed	Predicted	Observed	Predicted	Observed	Predicted
1	13588.77	11810.39	25162.33	24259.36	47411.46	47719.62	112476.4	109093.1
2	30343.71	31260.73	53520.67	56020.48	74880.79	74446.85	187925	184097.5

Table 3.59 Observed and predicted integration areas of hormones at several experimental conditions (continued).

3	24233.51	24653.79	40832.53	41595.58	66486.81	72049.57	157771.2	162763.3
4	24439.69	26946.42	47506.97	50945.74	68900.91	68373.19	166362.3	172044.8
5	12965.73	12843.02	27712.36	28565.82	50585.06	48300.64	129583.8	127370.2
6	26112.1	28055.57	43691.18	48033.68	78284.43	80348.01	162332.9	170764.4
7	17891.08	18907.48	27059.54	31294.61	53724.18	59952.39	127724	133927.7
8	19112.77	20447.41	37481.69	37075.31	64106.86	61969.92	140459.5	140843.2
9	16333.18	15273.8	22479.6	23861.07	56766.19	54867.3	120123.8	120440.8
10	18563.3	19354.93	35049.38	36263.64	62234.49	64974.67	147764.2	147906.7
R^2	0.958713		0.946327		0.938551		0.943031	
Adeq Precision	16.72297		13.59588		13.55452		13.18931	

Table 3.60 Possible optimized conditions to maximize integration area of hormones.

SN	SGP (arb)	ISGP (arb)	AGP (arb)	CT (°C)	VT (°C)	CGP (mTorr)	SV (V)	EE2	E1	E2	E3
1	**32.52**	**0.41**	**16.91**	**253.9**	**351.88**	**1.9**	**2740**	**36838**	**204298**	**58498**	**87257**
2	32.13	1.23	13.18	261.72	334.75	2.04	2650	36429	226297	62043	94747
3	33.52	1.15	11.25	252.28	336.1	1.65	2612	36345	220982	66104	91498
4	32.05	1.03	10.02	268.51	344.17	2.5	2589	35818	229343	59933	93251

The model equation can be used to evaluate variation of response with different factors. For three factors, it is necessary to keep one of the factors at constant value to be able plot 3D surface plots. In this case there are 7 factors and numerous numbers of plots can be obtained. In order to keep the process simple, only variation of integration area of hormones with ISGP and SGP were plotted. The other factors were kept constant at the optimized values. Figure 3.49 depicts the integration area of E1 for different values of ISGP and SGP. SGP has got more significant effect than ISGP on the response. The response significantly increases from around 180000 to over 210000 when SGP was decreased from SGP= 38 arb to SGP= 32 arb for ISGP between 0 arb and 2 arb. However, response varies around 170000 or 210000 for different ISGP at SGP= 38 arb and SGP= 32 arb, respectively. Almost the same effects of these factors can be observed for E2 (Figure 3.50) and E3. A slight increase in the response can be obtained when ISGP was decreased from 2 arb to 0 arb for any values of SGPs. But, decreasing SGP provides a substantial increase in the response.

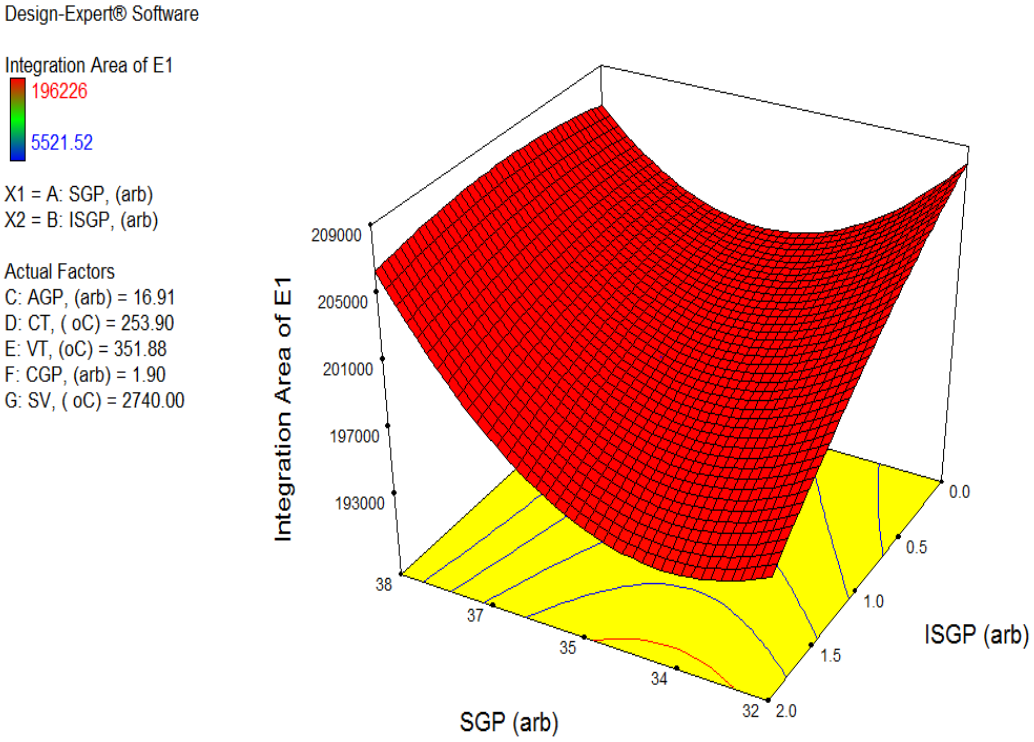


Figure 3.49 Surface plot of integration area of E1 at optimized condition (solution 1).

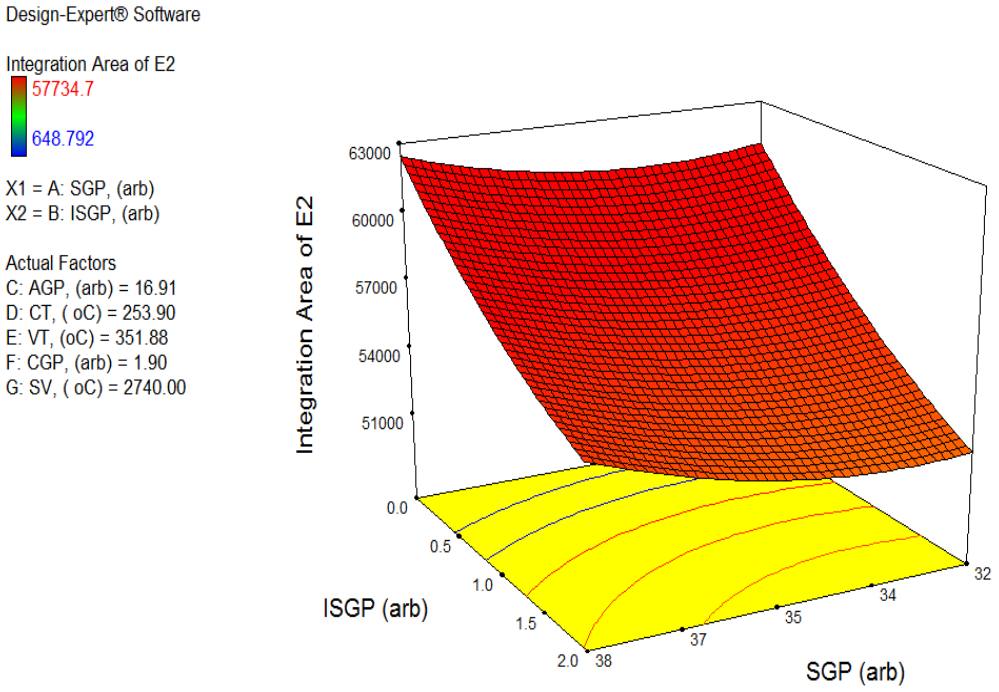


Figure 3.50 Surface plot of integration area of E2 at optimized condition (solution1).

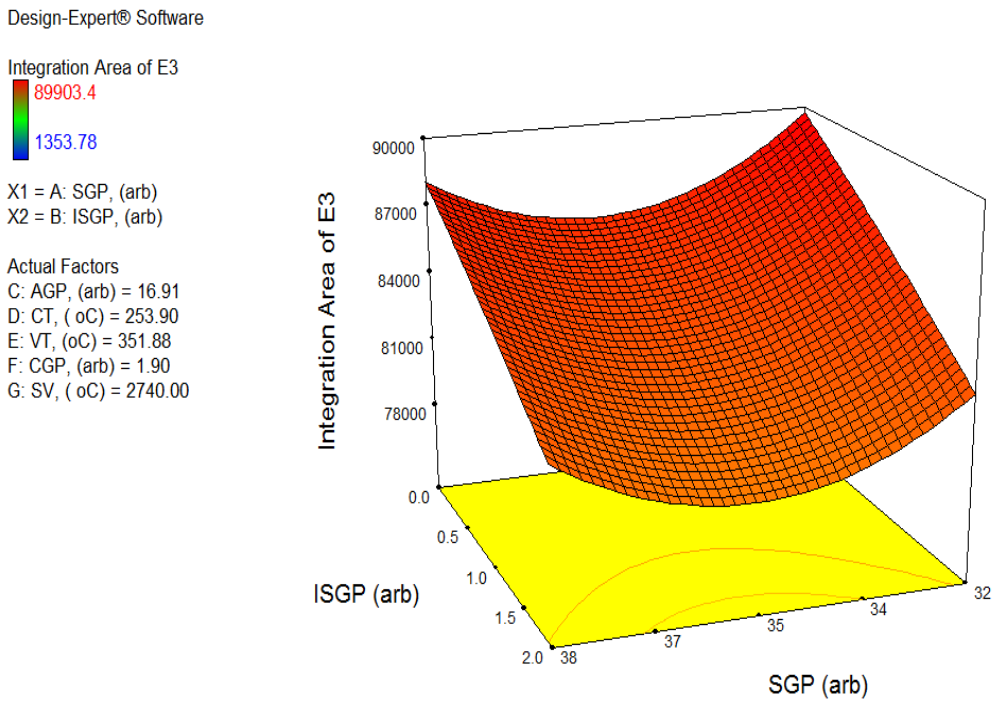


Figure 3.51 Surface plot of integration area of E3 at optimized condition (solution 1).

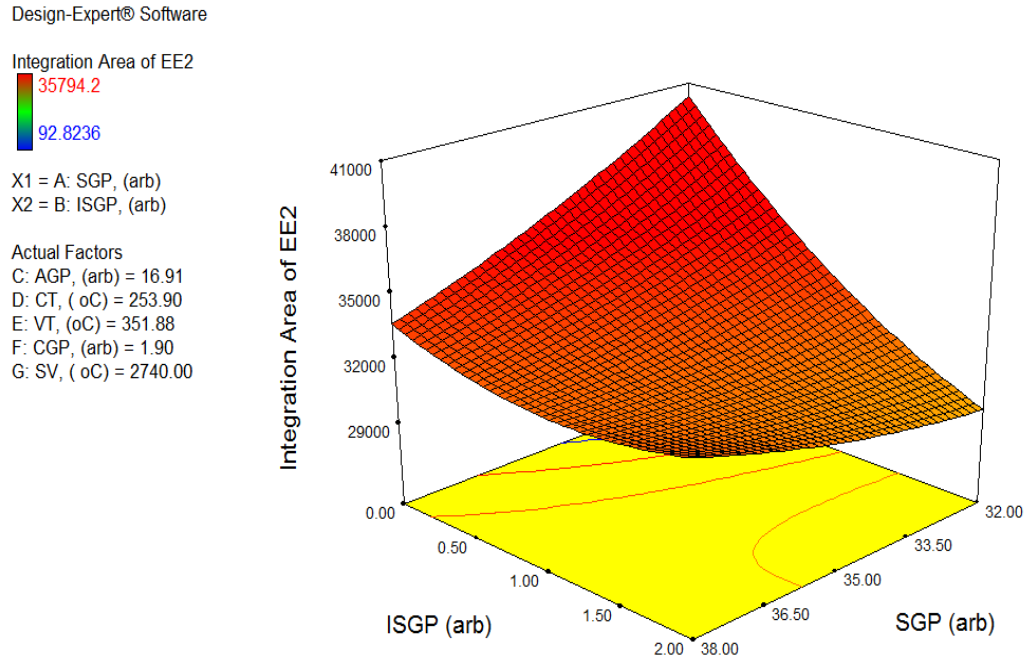


Figure 3.52 Surface plot of integration area of EE2 at optimized condition (solution 1).

The results were quite different for EE2. ISGP resulted in more significant effect on EE2 integration area than that of SGP. But, the interaction between factors was substantial as well, especially at low values of factors. The highest response area was

obtained as 40000 when SGP= 32 arb and ISGP= 0. However, it was around 30000 at SGP= 38 arb and ISGP= 2 arb. Table 3.61 depicts that verification point of Box-Behnken design and figure 3.53 depicts that chromatogram of prediction point of 3.

Table 3.61 Verification studies for Box-Behnken design.

Run	SGP (arb)	ISGP (arb)	AGP (arb)	CT (^{o}C)	VT (^{o}C)	CGP (mTorr)	SV (V)	Area (EE2) Predicted / Observed
prediction-1	38	1.5	12	325	350	1.5	2750	20375/ 22623
prediction-2	34	0.5	19	340	400	2.2	3000	4941/ 3769
prediction-3	33	0.4	17	254	352	1.9	2740	38023/ 39840

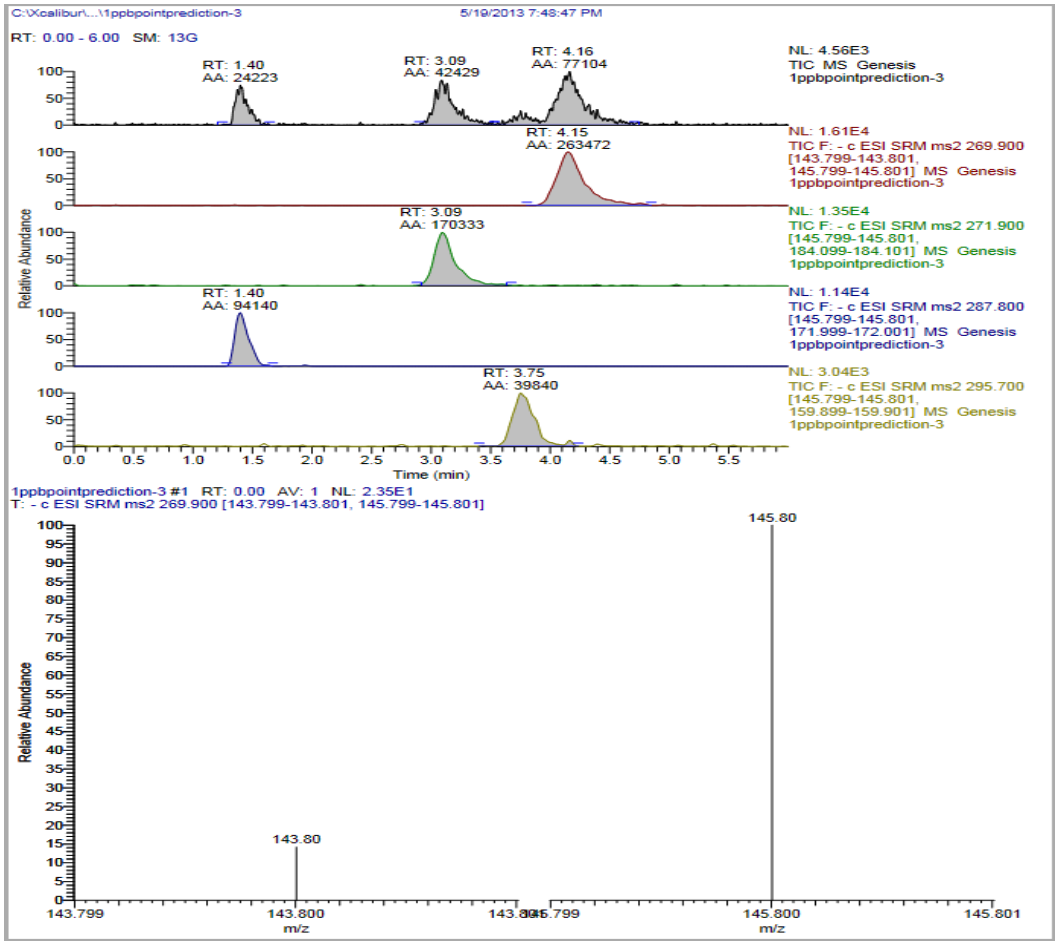


Figure 3.53 Depicts that chromatogram of verification point of 3.

3.6 Calibration Curve Studies

Calibration curves were constructed and shown in Figure 3.54(E1 and E2) and Figure 3.55 (E3 and EE2). Concentration ranges were fixed at 5 ng/L - 30 ng/L for E1, 75 ng/L - 1000 ng/L for E2, 10 ng/L - 75 ng/L for E3 and 75 ng/L - 250 ng/L for EE2. Regression coefficients were determined and these values are greater than acceptable value (0.97). This result indicated that there are linear relationship between concentrations and responses.

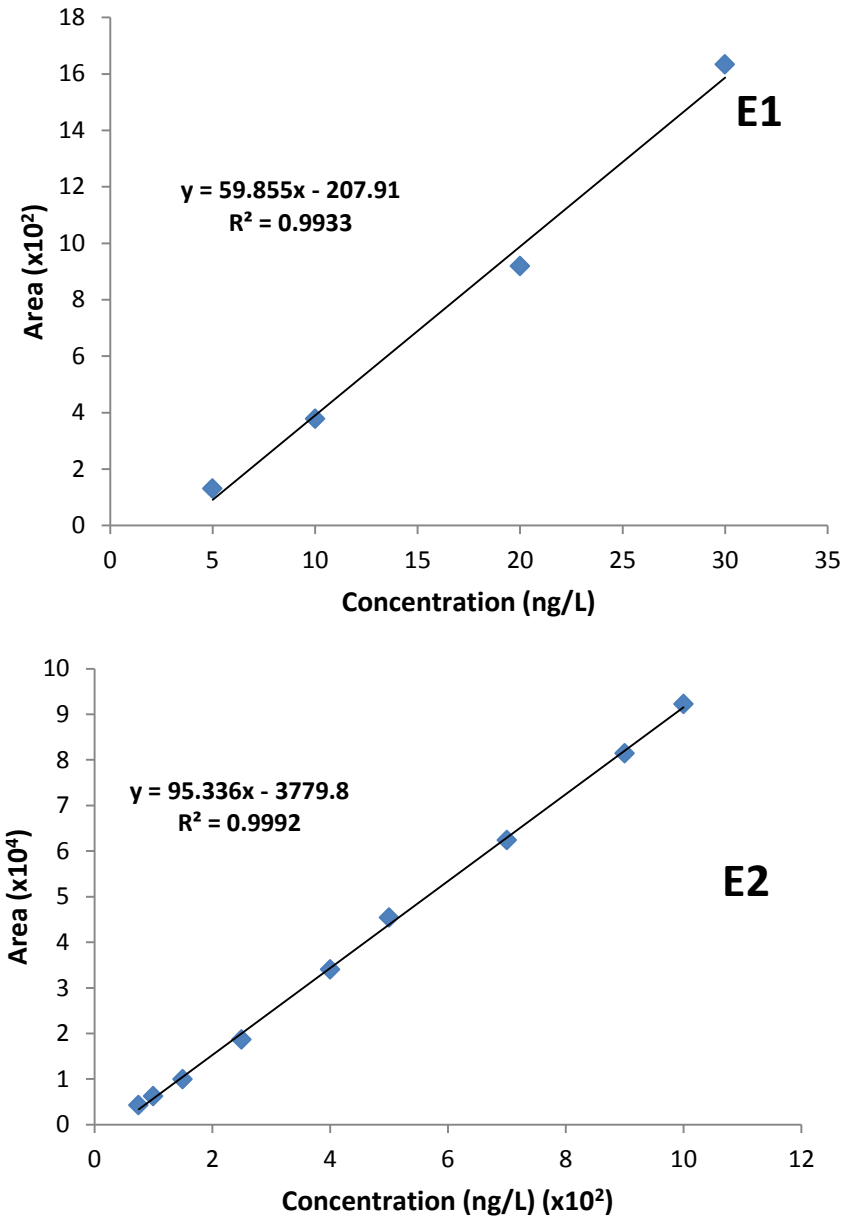
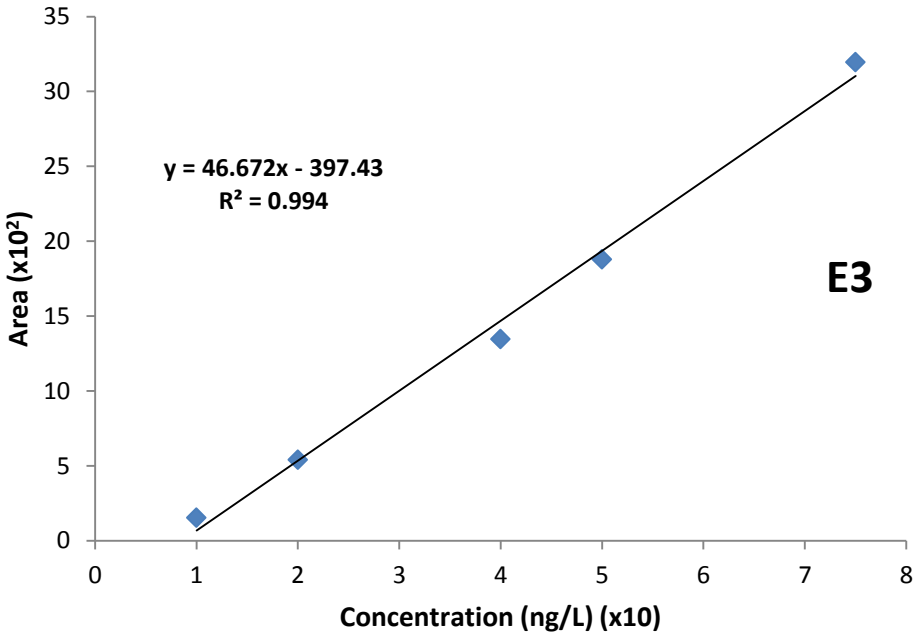


Figure 3.54 Calibration curves of E1 and E2.



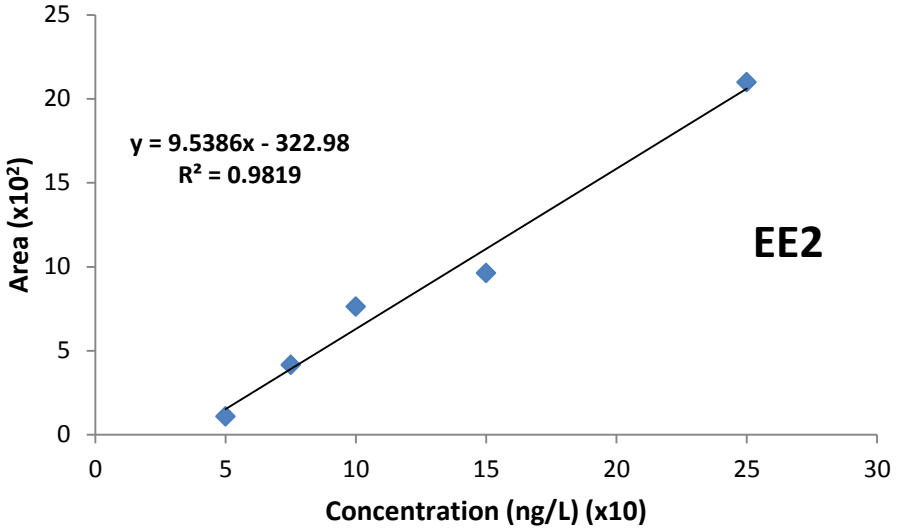


Figure 3.55 Calibration curves of E3 and EE2.

CHAPTER FOUR
CONCLUSIONS

Biological development of human being depends on closely related hormones reactions in the body. The interferences in hormone secretion and functions by different factors will lead to abnormalities in metabolism, reproduction and morphology of organisms. One of these factors is the chemicals called as Endocrine Disrupting Chemical (EDC). EDC's can exist in water, air, soil and wastes. They are one of the important environmental pollutants which extremely threaten the normal development of plants, animals and human being. The EDC's in domestic wastewater and generated by waste incineration, agricultural and industrial activities can be taken into the body through breathing, food chain and ingestion of water. These disruptors are perceived by the body as genuine hormones because they elicit the same chemical reactions as natural hormones, prevent naturally occurring hormones from affecting cells in the usual way by blocking the cell receptors; elicit unusual or abnormal reactions in cells. The synthetic EDCs are persistent, toxic and bio accumulative substances (persistent organic pollutants (POPs). Pesticides (aldrin, dieldrin, endrin, heptachlor etc), industrial chemicals (PCBs) and others chemicals such as dioxin, furan and PAH are within the group of synthetic EDC. The natural EDCs are known as estrogenic hormones as E1, E2, E3 and EE2 are generated by through body function of human or consumption of estrogen hormone for birth control. The existence of synthetic or natural ECDs chemical in air, soil and water has been detected and some adverse effects of them on the organisms even at nano gram level have been reported. Development of methods for measurement of very low concentration in water, wastewater and even in soil will be very helpful to advance the knowledge about their adverse health effect on human and animals. Moreover, it will also be helpful for the establishment of national or international control standards of these chemicals.

By considering these facts, the thesis aimed to develop an instrumental method for the measurement of estrogenic hormones as E1, E2, E3 and EE2 in LC-MS/MS. The pre-optimization of mobile phase, injection solution compositions and flow for peak

symmetry and resolution factor by using central composite design was conducted as initial stage of thesis. The optimized conditions were determined as % ACN_m= 40, %ACN_s= 25 and flow= 300 µL/min. The peak symmetry was at acceptable level around PS=1 but resolution factors were low around 0.6 to 1.7 depending on the hormone type. Low RF could have caused insufficient separation of peaks. In addition, integration area under these conditions were low as well. Ionization of hormones needed improvement. Alkaline addition as NH_4OH significantly improved integration area. Alkaline media support to increase ionization rate of hormones, because acidity constant of target analytes (pK_a) are around 10. This means that increasing alkalinity will have positive effect on ionization. The effect of %NH_4OH on ionization was studied between 0 to 17 %. Statistical analysis of results indicated that 3% NH_4OH was the optimum concentration to get highest integration area for all hormones.

The optimization studies should be carried out for narrow ranges of significant factors. There are 10 different LC-MS/MS running factors that could affect integration area of analyte. Factor selection was conducted by Single Factor Experimental Design method. The significant factors were sheath gas pressure, spray voltage, vaporizer temperature, cone position and collision gas pressure. On the other hand, capillary temperature, ion sweep gas pressure and aux gas pressure did not significantly affect the response area of all hormones. The pre-selected running conditions were SGP= 35 arb, SV= 2750 V, VT= 375 ^oC, cone position= D, CGP= 2 arb for significant factors. The maximum integration area achieved for E1 and E3, for example, by this experimental study were around A= 45000 and A= 18000, respectively.

Optimization of elution conditions for the new mobile phase with NH_4OH and pre-selected running conditions resulted in a substantial improvement in resolution factor although peak symmetry was at acceptable level. The final elution conditions were determined as % ACN_s= 28, % ACN_m=44, Flow=136.9 µL/min with corresponding RF values of hormones as E3=3.76, E2=1.25, EE2= 0.58. The most substantial improvement was achieved between E1 and E3. In addition EE2

separation in the chromatogram was sufficient compared to the pre-optimization studies. Total instrumental analysis time was achieved as 5 minutes by this second optimization.

MS/MS conditions were further optimized using response surface method. The interface conditions were determined as sheath gas= 33 arb, ion sweep gas= 0.4 arb, aux gas pressure= 17 arb, capillary temperature= 254 ^{o}C, vaporizer temperature= 352 ^{o}C, collision gas= 1.9 mTorr, spray voltage= 2740 V and cone position set at D. Substantial improvement in integration areas of hormones were achieved by this final optimization. The area for E1 and E3 increased up to E1= 200000 and for E3= 85000.

Calibration studies were performed and regression values coefficients were obtained at around 0.98 – 0.99. Linear range of calibration curve developed at the optimal conditions. It was revealed that even 1.6 ng/L of E1, 49 ng/L of E2, 3.2 ng/L of E3 and 23.2 ng/L of EE2 can be detected by the developed optimized conditions.

The measurement of these hormones still needs further studies as given below,

- Method development about preconcentration and purification techniques for measuring hormone concentrations, e.g., in wastewater, surface water and drinking water can be conducted. This study is being carried out by MSc. student Can Aftafa from Chemistry Department of EGE University.
- The method can be applied to water, wastewater and drinking water to investigate the matrix effect.
- Hormones are transformed to their conjugates through biochemical reactions in the body or in the water. Methods for the measurement of conjugates can be developed.
- Biological treatment of these hormones, process optimization and determination of alteration products after treatment can be studied.

- The concentrations of these hormones and their conjugates in the surface waters or in the effluent of domestic wastewater treatment plant can be monitored.
- The lowest concentration of hormones that could cause shift in the population from male to female of an aquatic animal can be investigated.

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APPENDICES

Table 1 Variation of integration area of E1 with NH_4OH concentration in mobile phase.

Replicate Number	% NH_4OH Concentration in Mobile Phase (200mM)						
	0	3	5	7	10	15	17
1	464999	757254	939542	742302	726983	710048	741178
2	378566	935188	435712	745196	729584	710533	744770
3	281782	682782	1028236	788230	767019	624417	716172
4	330897	690926	707873	768337	744723	611596	551245
5	372140	833536	1030780	747322	739401	642404	468470
6	381325	944411	1034792	757614	754575	660802	379633
7	378720	862915	1012192	756179	745538	699565	404157
8	410189	942813	1017491	749613	754882	710710	
9	409581	903930	732459	766516	753804	740855	
10	288265	850704	579708	745091	739183	722992	
Mean	369647	840446	851879	756640	745569	671259	572232
n	10	10	10	10	10	10	7
SD	56253.13	99491.41	221019.6	14296.66	12365.96	41576.93	161028.8

Table 2 LSD Comparison test for the different levels of NH_4OH concentration on integration area of E1.

Levels	$(\overline{Y}_i - \overline{Y}_j)\, t_{calculated}$	$t_{critic\ \ (\alpha\ .05)}$	$t_{calulated} > t_{critic}$ *
LSD test for comparing 0% NH_4OH with the other levels			
0%-3%	-470799	98938	Significant
0%-5%	-482232	98938	Significant
0%-7%	-386993	98938	Significant
%0-%10	-375923	98938	Significant
%0-%15	-301613	98938	Significant
%0-%17	-202585	109025	Significant
LSD test for comparing 3% NH_4OH with the other levels			
%3-%5	-11433	98938	Insignificant
%3-%7	83806	98938	Insignificant
%3-%10	94877	98938	Insignificant
%3-%15	169186	98938	Significant
%3-%17	268214	109025	Significant
LSD test for comparing 5% NH_4OH with the other levels			
%5-%7	95239	98938	Insignificant
%5-%10	106309	98938	Significant
%5-%15	180619	98938	Significant
%5-%17	279647	109025	Significant
LSD test for comparing 7% NH_4OH with the other levels			
%7-%10	11071	98938	Insignificant
%7-%15	85381	98938	Insignificant
%7-%17	184408	109025	Significant
LSD test for comparing 10% NH_4OH with the other levels			
%10-%15	74310	98938	Insignificant
%10-%17	173337	109025	Significant
LSD test for comparing 15% NH_4OH with the other levels			
%15-%17	99027	109025	Insignificant

*If $|t_{calculated}| > t_{critic}$, The difference between the means of the levels is significant.

Table 3 Variation of integration area of E2 with NH_4OH concentration in mobile phase.

Replicate Number	% NH_4OH Concentration in Mobile Phase (200mM)						
	0	3	5	7	10	15	17
1	266650.840	411942.271	414113.979	320831.881	305505.317	307150.116	310285.504
2	168255.512	307459.444		322877.637	317210.249	291923.002	310463.614
3	118764.560	305894.520	443356.976	334186.291	332901.398	261892.330	302782.276
4	144413.972	375774.274	321448.457	325699.139	326141.045		228925.831
5	155605.691	420033.011	448199.974	321537.523	311308.957	280692.856	
6	162581.663	416335.176	443136.043	326608.944	323937.107	283792.294	
7	171067.130	417939.639	435317.324	321080.121	317943.951	288987.180	
8	175158.490	426993.897	438959.723	321607.672	316103.617	306339.221	
9	172909.606	413963.786	324212.595	324303.991	320231.306	313115.389	
10	122585.231			325697.990	308831.900	306733.123	
Mean	165799.270	388481.780	408593.134	324443.119	318011.485	288682.428	288114.306
n	10	9	8	10	10	9	4
SD	40817.051	48565.320	53919.915	4028.725	8284.653	15646.448	39621.032

Table 4 LSD Comparison test for the different levels of NH_4OH concentration on integration area of E2.

Levels	$(\overline{Y}_i - \overline{Y}_j)$ $t_{calculated}$	t_{critic} $(\alpha .05)$	$t_{calulated} > t_{critic}$ *
LSD test for comparing 0% NH_4OH with the other levels			
0%-3%	-222682.510	42002.743	Significant
0%-5%	-242793.864	43362.405	Significant
0%-7%	-158643.849	30674.075	Significant
%0-%10	-152212.215	30674.075	Significant
%0-%15	-122883.159	42002.743	Significant
%0-%17	-122315.037	54082.421	Significant
LSD test for comparing 3% NH_4OH with the other levels			
%3-%5	-20111.354	33328.418	Insignificant
%3-%7	64038.661	42002.743	Significant
%3-%10	70470.295	42002.743	Significant
%3-%15	99799.352	32333.315	Significant
%3-%17	100367.473	41217.050	Significant
LSD test for comparing 5% NH_4OH with the other levels			
%5-%7	84150.015	43362.405	Significant
%5-%10	90581.649	43362.405	Significant
%5-%15	119910.706	33328.418	Significant
%5-%17	120478.828	54934.165	Significant
LSD test for comparing 7% NH_4OH with the other levels			
%7-%10	6431.634	30674.075	Insignificant
%7-%15	35760.691	42002.743	Insignificant
%7-%17	36328.813	54082.421	Insignificant
LSD test for comparing 10% NH_4OH with the other levels			
%10-%15	29329.056	42002.743	Insignificant
%10-%17	29897.178	54082.421	Insignificant
LSD test for comparing 15% NH_4OH with the other levels			
%15-%17	568.122	41217.050	Insignificant

*If $| t_{calculated} | > t_{critic}$, The difference between the means of the levels is significant.

Table 5 Variation of integration area of E3 with NH_4OH concentration in mobile phase.

Replicate Number	% NH_4OH Concentration in Mobile Phase (200mM)						
	0	3	5	7	10	15	17
1	250281	317984	347125	272197	257338	263263	270264
2	151572	255561	158297	273394	284495	264940	281593
3	107319	240930	380690	290793	283600	243928	260871
4	121330	292819	273567	279695	274287	238106	204341
5	138499	330085	362274	272469	274123	251780	180364
6	145962	327532	376062	281617	280401	261800	141514
7	148170	338619	359806	270016	264525	251574	156592
8	159472	329155	369843	274102	268919	272591	
9	159759	319720	280067	279617	273481	274747	
10	109762		213984	279851	265972	264484	
Mean	149213	305823	312171	277375	272714	255998	213648
n	10	9	10	10	10	10	7
SD	40349	35227	77419	6202	8723	11618	57307

Table 6 LSD Comparison test for the different levels of NH_4OH concentration on integration area of E3.

Levels	$(\overline{Y}_i - \overline{Y}_j)$ $t_{calculated}$	t_{critic} (α .05)	$t_{calulated} > t_{critic}$ *
LSD test for comparing 0% NH_4OH with the other levels			
0%-3%	-156610	37956	Significant
0%-5%	-162959	36944	Significant
0%-7%	-128162	36944	Significant
%0-%10	-123502	36944	Significant
%0-%15	-106785	36944	Significant
%0-%17	-64436	40710	Significant
LSD test for comparing 3% NH_4OH with the other levels			
%3-%5	-6349	37956	Insignificant
%3-%7	28448	37956	Insignificant
%3-%10	33109	37956	Insignificant
%3-%15	49825	37956	Significant
%3-%17	92175	41631	Significant
LSD test for comparing 5% NH_4OH with the other levels			
%5-%7	34796	36944	Insignificant
%5-%10	39457	36944	Significant
%5-%15	56174	36944	Significant
%5-%17	98523	40710	Significant
LSD test for comparing 7% NH_4OH with the other levels			
%7-%10	4661	36944	Insignificant
%7-%15	21377	36944	Insignificant
%7-%17	63727	40710	Significant
LSD test for comparing 10% NH_4OH with the other levels			
%10-%15	16716	36944	Insignificant
%10-%17	59066	40710	Significant
LSD test for comparing 15% NH_4OH with the other levels			
%15-%17	42349	40710	Significant

*If $| t_{calculated} | > t_{critic}$, The difference between the means of the levels is significant.

Table 7 Variation of integration area of EE2 with NH_4OH concentration in mobile phase.

Replicate Number	% NH_4OH Concentration in Mobile Phase (200mM)						
	0	3	5	7	10	15	17
1	73813	104960	142431	106881	99606	104318	112743
2	52407	134386	55029	103299	103731	107809	104133
3	37542	105472	151069	111530	108315	90161	99297
4	46319	99793	105304	106455	106735	89838	82633
5	49863	121360	144908	106691	102667	90614	62811
6	53093	136748	145557	110556	101796	100766	52055
7	55107	137495	143760	107513	106153	99696	58714
8	55587	137349	149070	102847	106536	106394	
9	53071	144934	102924	104537	109605	113022	
10	39757	140571	74080	104209	104427	109372	
Mean	51656	124722	121413	106452	104957	98699	81769
n	10	10	10	10	10	10	7
SD	9974.542	17186,64	34911,07	2898,947	3084,494	7519,808	24294,64

Table 8 LSD Comparison test for the different levels of NH_4OH concentration on integration area of EE2.

Levels	$(\bar{Y}_i - \bar{Y}_j)$ $t_{calculated}$	t_{critic} (α .05)	$t_{calulated} > t_{critic}$ *
LSD test for comparing 0% NH_4OH with the other levels			
0%-3%	-73066	15836	Significant
0%-5%	-69757	15836	Significant
0%-7%	-54796	15836	Significant
%0-%10	-53301	15836	Significant
%0-%15	-47043	15836	Significant
%0-%17	-30113	17451	Significant
LSD test for comparing 3% NH_4OH with the other levels			
%3-%5	3308	15836	Insignificant
%3-%7	18270	15836	Significant
%3-%10	19765	15836	Significant
%3-%15	26023	15836	Significant
%3-%17	42952	17451	Significant
LSD test for comparing 5% NH_4OH with the other levels			
%5-%7	14962	15836	Insignificant
%5-%10	16456	15836	Significant
%5-%15	22714	15836	Significant
%5-%17	39644	17451	Significant
LSD test for comparing 7% NH_4OH with the other levels			
%7-%10	1495	15836	Insignificant
%7-%15	7753	15836	Insignificant
%7-%17	24682	17451	Significant
LSD test for comparing 10% NH_4OH with the other levels			
%10-%15	6258	15836	Insignificant
%10-%17	23188	17451	Significant
LSD test for comparing 15% NH_4OH with the other levels			
%15-%17	16930	17451	Insignificant

*If $|t_{calculated}| > t_{critic}$, The difference between the means of the levels is significant.

Table 9 Raw data for the effect of sheath gas on integration area of hormones

Number of Replicate	E1			E2			E3			EE2		
	Levels of Sheath Gas			Levels of Sheath Gas			Levels of Sheath Gas			Levels of Sheath Gas		
	sg -30	sg-35	sg-40	sg -30	sg-35	sg-40	sg -30	sg-35	sg-40	sg -30	sg-35	sg-40
1	14320	24833	15665	2522	5772	2649	11137	10187	8042	1645	1690	726
2	19279	22781	12450	2357	4811	2477	5601	10021	5016	1405	2444	916
3	17253	25135	16633	3028	5431	3016	6209	9898	6768	363	1620	1915
4		23639			6969		7106	9958	5248	565	2164	923
5												
Mean	16951	24097	14916	2636	5746	2714	7513	10016	6268	994	1979	1120
n	3	4	3	3	4	3	4	4	4	4	4	4
SD	2493.247	1089.474	2189.492	349.90498	907.44842	275.4727	2493.6437	124.44888	1415.0393	625.4645	393.10808	537.82612

Table 10 Raw data for the effect of spray voltage on integration area of E1 and E2.

Replicate number	E1							E2						
	Levels of Spray Voltage							Levels of Spray Voltage						
	sv-2000	sv-2250	sv-2750	sv-3000	sv-3250	sv-3500	sv-3750	sv-2000	sv-2250	sv-2750	sv-3000	sv-3250	sv-3500	sv-3750
1	10062	21625	25394	26250	22500	12586	13713	3060	3965	4961	4549	6060	2456	1187
2	14999	27366	18460	28956	23110	14475	10668	4389	4734	4077	5679	5437	3052	1325
3	10847	15404	33758	27530	22866	12282	9387	3114	3189	8124	4894	4217	2998	3161
4	19360	21287	33833	21096	26930	17876	10164	2919	5548	8690	6358	4589	2963	3129
5	16447	21595	33181	17134	21102	20745	10041	4032	4869	8583	3609	2730	4644	925
Mean	14343	21455	28925	24193	23302	15593	10795	3503	4461	6887	5018	4607	3223	1946
n	5	5	5	5	5	5	5	5	5	5	5	5	5	5
SD	3891.48	4231.87	6847.46	4936.46	2172.19	3640.27	1693.94	661.9919	906.2476	2194.473	1054.902	1272.302	829.8406	1104.655

Table 11 Raw data for the effect of spray voltage on integration area of E3 and EE2.

Replicate number	E3							EE2						
	Levels of Spray Voltage							Levels of Spray Voltage						
	sv-2000	sv-2250	sv-2750	sv-3000	sv-3250	sv-3500	sv-3750	sv-2000	sv-2250	sv-2750	sv-3000	sv-3250	sv-3500	sv-3750
1	3654	9360	10116	7070	7123	5572	5543	752	3344	1104	1419	1706	989	796
2	2791	7640	7212	9517	8606	5749	5200	1333	2124	1805	814	1759	1421	67
3	3502	5699	10064	9037	9829	5413	4137	1596	2440	2669	2154	2196	390	1080
4	4722	8578	12002	13989	7186	6472	3271	1174	1845	3159	2200	784	966	415
5	4150	7041	10796	5030	7581	8669	3628	1009	2073	1128	1611	2115	2310	684
Mean	3764	7664	10038	8928	8065	6375	4356	1173	2365	1973	1640	1712	1215	608
n	5	5	5	5	5	5	5	5	5	5	5	5	5	5
SD	723.52	1410.82	1762.57	3339.81	1151.05	1344.54	984.61	319.88	586.96	920.23	572.21	561.43	713.41	385.06

Table 12 Raw data for the effect of vaporizer temperature on integration area of E1 and E2.

Replicate number	E1								E2							
	Levels of Vaporizer Temperature								Levels of Vaporizer Temperature							
	vt-200	vt-250	vt-300	vt-350	vt-375	vt-400	vt-425	vt-450	vt-200	vt-250	vt-300	vt-350	vt-375	vt-400	vt-425	vt-450
1	18062	19526	33581	32366	62048	60431	35786	31495	4495.50	6624.88	4797.43	8444.76	7123.48	4902.16	6786.38	4860.19
2	22416	22770	28965	32434	64086	64699	35014	31850	4119.82	5035.52	4204.16	6753.23	7519.25	7430.91	6841.89	5030.81
3	19982	20104	26174	32871	64747	67035	31629	33825	5397.34	3164.08	5128.32	6033.56	10278.39	7821.64	5994.42	7851.17
4	17245	35181	33062	35872	60649	31560	36127	53237	3044.12	8213.61	6070.81	6327.78	8966.36	8894.19	7020.30	3508.22
5	19456	23524	31295	33350	63428	34525	35486	49463	2894.44	5348.81	6995.19	7685.89	7701.63	9724.43	5854.00	6817.76
Mean	19432	24221	30616	33379	62992	51650	34808	39974	3990.25	5677.38	5439.18	7049.04	8317.82	7754.66	6499.40	5613.63
n	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
SD	1992	6358	3070	1449	1647	17183	1824	10508	1042.58	1881.94	1101.58	999.06	1295.02	1831.98	534.45	1717.31

Table 13 Raw data for the effect of vaporizer temperature on integration area of E3 and EE2.

Replicate number	E3								EE2							
	Levels of Vaporizer Temperature								Levels of Vaporizer Temperature							
	vt-200	vt-250	vt-300	vt-350	vt-375	vt-400	vt-425	vt-450	vt-200	vt-250	vt-300	vt-350	vt-375	vt-400	vt-425	vt-450
1	9191	6486	11843	14625	25444	25272	16938	14442	1710	1653	2930	2511	2680	3911	3086	2578
2	6958	8326	11433	13510	26041	26723	16479	11091	1226	1419	3764	3711	4324	2345	3103	2850
3	5993	8548	12689	14485	26632	23555	16402	11386	616	1898	2734	3100	4233	4458	2935	1611
4	7521	11704	13526	11545	22936	17634	12634	20901	2147	1375	4006	3228	4388	2311	1872	1743
5	5102	9208	12983	14162	28431	16898	16750	20814	1012	1678	2173	4440	4797	4020	2752	1712
Mean	6953	8854	12495	13665	25897	22017	15841	15727	1342	1605	3121	3398	4084	3409	2750	2099
n	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
SD	1555	1886	850	1261	1997	4487	1805	4864	599	213	755	723	814	1008	510	572

Table 14 Raw data for the effect of aux gas pressure on integration area of all hormones.

	E1				E2				E3				EE2			
Replicate number	Levels of Aux Gas Pressure				Levels of Aux Gas Pressure				Levels of Aux Gas Pressure				Levels of Aux Gas Pressure			
	ag-5	ag-10	ag-15	ag-20	ag-5	ag-10	ag-15	ag-20	ag-5	ag-10	ag-15	ag-20	ag-5	ag-10	ag-15	ag-20
1	36113	34794	39657	34409	6535	7218	10806	10026	13638	12903	14378	13486	4905	2311	1852	909
2	33844	34913	38701	34280	8690	7114	9213	7180	15768	12661	16993	19744	1843	3506	3303	3967
3	35548	29862	35905	36682	7452	10269	5793	10148	14748	15619	15159	22962	2311	2263	2731	4682
4	33671	34684	40776	33067	9289	6518	6637	6392	16759	14956	14262	15347	4838	3236	3823	1517
5	36332	37577	33593	36667	7499	8007	9125	10751	13456	13873	14153	14558	2172	3964	3400	1452
Mean	35102	34366	37727	35021	7893	7825	8315	8899	14874	14003	14989	17219	3214	3056	3022	2505
n	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
SD	1261	2792	2932	1598	1093	1465	2052	1968	1406	1279	1188	3996	1523	749	761	1696

Table 15 Raw data for the effect of capillary temperature on integration area of all hormones.

Number of Replicate	E1			E2			E3			EE2		
	Levels of Capillary Temperature			Levels of Capillary Temperature			Levels of Capillary Temperature			Levels of Capillary Temperature		
	c.tem 300	c.tem 325	c.tem 350	c.tem 300	c.tem 325	c.tem 350	c.tem 300	c.tem 325	c.tem 350	c.tem 300	c.tem 325	c.tem 350
1	35583	34167	31521	5112	5921	4882	14556	14395	15077	2939	2995	2049
2	32600	38582	29341	9318	6970	5707	14243	11623	12457	4543	1898	2896
3	41218	33995	31577	11490	4994	5169	11718	14671	11122	3236	2912	3118
4	53120	32753	31584	9808	6235	5984	22082	13612	12168	4041	3887	1497
5												
Mean	40630	34874	31006	8932	6030	5436	15650	13575	12706	3690	2923	2390
n	4	4	4	4	4	4	4	4	4	4	4	4
SD	9061	2551	1110	2711	819	500	4472	1376	1682	735	814	753

Table 16 Raw data for the effect of ion sweep gas pressure on integration area of all hormones.

Number of Replicate	E1			E2			E3			EE2		
	Ion sweep gas pressure (arb)			Ion sweep gas pressure (arb)			Ion sweep gas pressure (arb)			Ion sweep gas pressure (arb)		
	is-0.5	is-0	is-1	is-0.5	is-0	is-1	is-0.5	is-0	is-1	is-0.5	is-0	is-1
1	36871	41205	38335	11352	10286	8019	17059	12694	13553	3936	5027	3070
2	41203	34777	39322	9936	9353	9052	16420	17901	15037	3967	2997	2546
3	37113	35299	36081	9476	9142	8785	16618	17238	16977	3216	4658	3049
4	38624	37866	32571	7217	11189	7767	12455	17969	17256	3976	2999	2095
5												
Mean	38453	37287	36577	9495	9992	8406	15638	16450	15706	3774	3920	2690
n	4	4	4	4	4	4	4	4	4	4	4	4
SD	1991	2941	2996	1716	940	611	2139	2526	1742	372	1075	465

Table 17 Raw data for the effect of cone position on integration area of all hormones.

Number of Replicate	E1			E2			E3			EE2		
	Cone Position			Cone Position			Cone Position			Cone Position		
	B	C	D	B	C	D	B	C	D	B	C	D
1	24340	39605	41380	7494	9550	10368	12860	17034	21503	3733	3573	4084
2	33557	42713	42701	6842	9938	12651	9890	17005	23572	3292	3567	5091
3	30067	43072	43994	7821	10888	9624	13288	17327	21324	4044	3208	3684
4	24533	38188	44619	7614	10451	12309	13568	18340	20050	3532	5153	5800
5												
Mean	28124	40895	43173	7442	10207	11238	12402	17426	21612	3650	3875	4664
n	4	4	4	4	4	4	4	4	4	4	4	4
SD	4491	2383	1438	423	585	1473	1699	626	1458	318	869	961

Table 18 Raw data for the effect of collision gas pressure (m Torr) on integration area of all hormones.

Number of Replicate	E1			E2			E3			EE2		
	Collision gas pressure (m Torr)			Collision gas pressure (m Torr)			Collision gas pressure (m Torr)			Collision gas pressure (m Torr)		
	cg-1	cg-1.5	cg-2	cg-1.5	cg-1	cg-2	cg-1.5	cg-1	cg-2	cg-1.5	cg-1	cg-2
1	18184	39624	43147	6246	2796	10375	15501	8456	17531	3246	1796	4903
2	18054	42797	43681	7225	2996	9722	14093	8203	16429	3913	1806	1772
3	18006	40233	40385	6449	4884	9377	18651	9390	18476	4793	2244	3677
4	19786	38884	39998	10828	4918	9295	15351	8484	14553	3959	2400	3585
5												
Mean	18507	40384	41803	7687	3898	9692	15899	8633	16747	3978	2061	3484
n	4	4	4	4	4	4	4	4	4	4	4	4
SD	856	1700	1880	2136	1161	491	1940	520	1685	633	307	1290

Table 19 Raw data of LSD test for the effect of spray voltage on integration area of E1.

Y_i vs Y_j	$(\overline{Y}_i - \overline{Y}_j)$ $t_{calculated}$	t_{critic} (α .05)	$t_{calulated} > t_{critic}$ *
2000 vs 2250	-7112	5476.7502	Significant
2000 vs 2750	-14582	5476.7502	Significant
2000 vs 3000	-9850	5476.7502	Significant
2000 vs 3250	-8959	5476.7502	Significant
2000 vs 3500	-1250	5476.7502	Insignificant
2000 vs 3750	3548	5476.7502	Insignificant
2250 vs 2750	-7470	5476.7502	Significant
2250 vs 3000	-2738	5476.7502	Insignificant
2250 vs 3250	-1846	5476.7502	Insignificant
2250 vs 3500	5863	5476.7502	Significant
2250 vs 3750	10661	5476.7502	Significant
2750 vs 3000	4732	5476.7502	Insignificant
2750 vs 3250	5624	5476.7502	Significant
2750 vs 3500	13332	5476.7502	Significant
2750 vs 3750	18131	5476.7502	Significant
3000 vs 3250	892	5476.7502	Insignificant
3000 vs 3500	8600	5476.7502	Significant
3000 vs 3750	13399	5476.7502	Significant
3250 vs 3500	7709	5476.7502	Significant
3250 vs 3750	12507	5476.7502	Significant
3500 vs 3750	4798	5476.7502	Insignificant

*If $|t_{calculated}| > t_{critic}$, The difference between the means of the levels is significant.

Table 20 Raw data of LSD test for the effect of spray voltage on integration area of E2.

Levels	$(\overline{Y}_i - \overline{Y}_j)$ $t_{calculated}$	t_{critic} (α .05)	$t_{calulated} > t_{critic}$ *
2000 vs 2250	-958	1603	Insignificant
2000 vs 2750	-3384	1603	Significant
2000 vs 3000	-1515	1603	Insignificant
2000 vs 3250	-1104	1603	Insignificant
2000 vs 3500	280	1603	Insignificant
2000 vs 3750	1557	1603	Insignificant
2250 vs 2750	-2426	1603	Significant
2250 vs 3000	-557	1603	Insignificant
2250 vs 3250	-146	1603	Insignificant
2250 vs 3500	1238	1603	Insignificant
2250 vs 3750	2515	1603	Significant
2750 vs 3000	1869	1603	Significant
2750 vs 3250	2280	1603	Significant
2750 vs 3500	3664	1603	Significant
2750 vs 3750	4942	1603	Significant
3000 vs 3250	411	1603	Insignificant
3000 vs 3500	1795	1603	Significant
3000 vs 3750	3072	1603	Significant
3250 vs 3500	1384	1603	Insignificant
3250 vs 3750	2661	1603	Significant
3500 vs 3750	1277	1603	Insignificant

*If $|t_{calculated}| > t_{critic}$, The difference between the means of the levels is significant.

Table 21 Raw data of LSD test for the effect of spray voltage on integration area of E3.

Levels	$(\overline{Y}_i - \overline{Y}_j)$ $t_{calculated}$	t_{critic} (α .05)	$t_{calulated} > t_{critic}$ *
2000 vs 2250	-3900	2237	Significant
2000 vs 2750	-6274	2237	Significant
2000 vs 3000	-5165	2237	Significant
2000 vs 3250	-4301	2237	Significant
2000 vs 3500	-2611	2237	Significant
2000 vs 3750	-592	2237	Insignificant
2250 vs 2750	-2374	2237	Significant
2250 vs 3000	-1265	2237	Insignificant
2250 vs 3250	-401	2237	Insignificant
2250 vs 3500	1288	2237	Insignificant
2250 vs 3750	3308	2237	Significant
2750 vs 3000	1109	2237	Insignificant
2750 vs 3250	1973	2237	Insignificant
2750 vs 3500	3663	2237	Significant
2750 vs 3750	5682	2237	Significant
3000 vs 3250	864	2237	Insignificant
3000 vs 3500	2553	2237	Significant
3000 vs 3750	4573	2237	Significant
3250 vs 3500	1690	2237	Insignificant
3250 vs 3750	3709	2237	Significant
3500 vs 3750	2019	2237	Insignificant

*If $|t_{calculated}| > t_{critic}$, The difference between the means of the levels is significant.

Table 22 Raw data of LSD test for the effect of spray voltage on integration area of EE2.

Levels	$(\overline{Y}_i - \overline{Y}_j)$ $t_{calculated}$	t_{critic} (α .05)	$t_{calulated} > t_{critic}$ *
2000 vs 2250	-1193	788	Significant
2000 vs 2750	-800	788	Significant
2000 vs 3000	-467	788	Insignificant
2000 vs 3250	-539	788	Insignificant
2000 vs 3500	-43	788	Insignificant
2000 vs 3750	564	788	Insignificant
2250 vs 2750	392	788	Insignificant
2250 vs 3000	726	788	Insignificant
2250 vs 3250	653	788	Insignificant
2250 vs 3500	1150	788	Significant
2250 vs 3750	1757	788	Significant
2750 vs 3000	333	788	Insignificant
2750 vs 3250	261	788	Insignificant
2750 vs 3500	758	788	Insignificant
2750 vs 3750	1365	788	Significant
3000 vs 3250	-72	788	Insignificant
3000 vs 3500	424	788	Insignificant
3000 vs 3750	1031	788	Significant
3250 vs 3500	497	788	Insignificant
3250 vs 3750	1104	788	Significant
3500 vs 3750	607	788	Insignificant

*If $|t_{calculated}| > t_{critic}$, The difference between the means of the levels is significant.

Table 23 Raw data of LSD test for the vaporizer temperature on integration area of E1.

Levels	$(\overline{Y}_i - \overline{Y}_j)$ $t_{calculated}$	t_{critic} (α .05)	$t_{calulated} > t_{critic}$ *
200 vs 250	-4789	9853	Insignificant
200 vs 300	-11183	9853	Significant
200 vs 350	-13946	9853	Significant
200 vs 375	-43559	9853	Significant
200 vs 400	-32218	9853	Significant
200 vs 425	-15376	9853	Significant
200 vs 450	-20542	9853	Significant
250 vs 300	-6395	9853	Insignificant
250 vs 350	-9158	9853	Insignificant
250 vs 375.	-38771	9853	Significant
250 vs 400	-27429	9853	Significant
250 vs 425	-10587	9853	Significant
250 vs 450	-15753	9853	Significant
300 vs 350	-2763	9853	Insignificant
300 vs 375	-32376	9853	Significant
300 vs 400	-21035	9853	Significant
300 vs 425	-4193	9853	Insignificant
300 vs 450	-9359	9853	Insignificant
350 vs 375	-29613	9853	Significant
350 vs 400	-18271	9853	Significant
350 vs 425	-1430	9853	Insignificant
350 vs 450	-6595	9853	Insignificant
375 vs 400	11342	9853	Significant
375 vs 425	28183	9853	Significant
375 vs 450	23018	9853	Significant
400 vs 425	16842	9853	Significant
400 vs 450	11676	9853	Significant
425 vs 450	-5166	9853	Insignificant

*If $|t_{calculated}| > t_{critic}$, The difference between the means of the levels is significant.

Table 24 Raw data of LSD test for the vaporizer temperature on integration area of E2.

Levels	$(\overline{Y}_i - \overline{Y}_j)$ $t_{calculated}$	t_{critic} (α .05)	$t_{calulated} > t_{critic}$ *
200 vs 250	-1687.13	1771.15	Insignificant
200 vs 300	-1448.93	1771.15	Insignificant
200 vs 350	-3058.80	1771.15	Significant
200 vs 375	-4327.57	1771.15	Significant
200 vs 400	-3764.42	1771.15	Significant
200 vs 425	-2509.15	1771.15	Significant
200 vs 450	-1623.38	1771.15	Insignificant
250 vs 300	238.20	1771.15	Insignificant
250 vs 350	-1371.66	1771.15	Insignificant
250 vs 375.	-2640.44	1771.15	Significant
250 vs 400	-2077.28	1771.15	Significant
250 vs 425	-822.02	1771.15	Insignificant
250 vs 450	63.75	1771.15	Insignificant
300 vs 350	-1609.86	1771.15	Insignificant
300 vs 375	-2878.64	1771.15	Significant
300 vs 400	-2315.48	1771.15	Significant
300 vs 425	-1060.22	1771.15	Insignificant
300 vs 450	-174.45	1771.15	Insignificant
350 vs 375	-1268.78	1771.15	Insignificant
350 vs 400	-705.62	1771.15	Insignificant
350 vs 425	549.64	1771.15	Insignificant
350 vs 450	1435.41	1771.15	Insignificant
375 vs 400	563.16	1771.15	Insignificant
375 vs 425	1818.42	1771.15	Significant
375 vs 450	2704.19	1771.15	Significant
400 vs 425	1255.27	1771.15	Insignificant
400 vs 450	2141.03	1771.15	Significant
425 vs 450	885.77	1771.15	Insignificant

*If $|t_{calculated}| > t_{critic}$, The difference between the means of the levels is significant.

Table 25 Raw data of LSD test for the vaporizer temperature on integration area of E3.

Levels	$(\overline{Y}_i - \overline{Y}_j)$ $t_{calculated}$	t_{critic} (α .05)	$t_{calulated} > t_{critic}$ *
200 vs 250	-1901	3510	Insignificant
200 vs 300	-5542	3510	Significant
200 vs 350	-6713	3510	Significant
200 vs 375	-18944	3510	Significant
200 vs 400	-15064	3510	Significant
200 vs 425	-8888	3510	Significant
200 vs 450	-8774	3510	Significant
250 vs 300	-3641	3510	Significant
250 vs 350	-4811	3510	Significant
250 vs 375.	-17042	3510	Significant
250 vs 400	-13162	3510	Significant
250 vs 425	-6986	3510	Significant
250 vs 450	-6873	3510	Significant
300 vs 350	-1171	3510	Significant
300 vs 375	-13402	3510	Significant
300 vs 400	-9522	3510	Significant
300 vs 425	-3346	3510	Insignificant
300 vs 450	-3232	3510	Insignificant
350 vs 375	-12231	3510	Significant
350 vs 400	-8351	3510	Significant
350 vs 425	-2175	3510	Insignificant
350 vs 450	-2061	3510	Insignificant
375 vs 400	3880	3510	Significant
375 vs 425	10056	3510	Significant
375 vs 450	10170	3510	Significant
400 vs 425	6176	3510	Significant
400 vs 450	6290	3510	Significant
425 vs 450	114	3510	Insignificant

*If $|t_{calculated}| > t_{critic}$, The difference between the means of the levels is significant.

Table 26 Raw data of LSD test for the vaporizer temperature on integration area of EE2.

Levels	$(\overline{Y}_i - \overline{Y}_j)$ $t_{calculated}$	t_{critic} (α .05)	$t_{calulated} > t_{critic}$ *
200 vs 250	-262	884	Insignificant
200 vs 300	-1779	884	Significant
200 vs 350	-2056	884	Significant
200 vs 375	-2742	884	Significant
200 vs 400	-2067	884	Significant
200 vs 425	-1407	884	Significant
200 vs 450	-757	884	Insignificant
250 vs 300	-1517	884	Significant
250 vs 350	-1794	884	Significant
250 vs 375.	-2479	884	Significant
250 vs 400	-1804	884	Significant
250 vs 425	-1145	884	Significant
250 vs 450	-494	884	Insignificant
300 vs 350	-277	884	Insignificant
300 vs 375	-963	884	Significant
300 vs 400	-288	884	Insignificant
300 vs 425	372	884	Insignificant
300 vs 450	1022	884	Significant
350 vs 375	-686	884	Insignificant
350 vs 400	-11	884	Insignificant
350 vs 425	649	884	Insignificant
350 vs 450	1299	884	Significant
375 vs 400	675	884	Insignificant
375 vs 425	1335	884	Significant
375 vs 450	1985	884	Significant
400 vs 425	659	884	Insignificant
400 vs 450	1310	884	Significant
425 vs 450	651	884	Insignificant

*If $|t_{calculated}| > t_{critic}$, The difference between the means of the levels is significant.