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INSTITUTE OF MEDICAL SCIENCES
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Güneş YILDIRIM
20103030001

MASTER'S PROGRAM THESIS

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Yüksek lisans öğrencisi Güneş YILDIRIM' ın çalışması jürimiz tarafından Farmasötik Kimya Anabilim Dalı yüksek lisans tezi olarak uygun görülmüştür.

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Başkan : Prof. Dr. Hülya AKGÜN

Üniversitesi : Yeditepe Üniversitesi

Üye : Prof. Dr. Durişehvar ÖZER ÜNAL

Üniversitesi : İstanbul Üniversitesi

Tez Danışmanı : Yrd. Doç. Dr. Ebru TÜRKÖZ ACAR

Üniversitesi : Yeditepe Üniversitesi

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Prof. Dr. Selçuk Yılmaz

ABSTRACT

Nowadays, the usage of clean-hygiene products as fast moving consumer goods which present a rapid solution is widely increasing. The most popular products of all these are wet wipes for hand cleaning. Wet wipes are produced for variety purpose such as baby cleaning, surface cleaning. Also, there are antibacterial types of wet wipes for hand cleaning. Benzalkonium chloride, benzethonium chloride, cetrimide, chlorhexidine, hexachlorophene, alcohol, hydrogen peroxide, hexamine hippurate, triclosan, cetylpyridinium chloride, dequalinium chloride are used as antibacterial agents in wet wipes. Benzalkonium chloride is quaternary ammonium surfactant, which widely used in health care and cosmetic preparations and it is also used as topical antiseptic and medical equipment disinfectants. Benzalkonium chloride which is a mixture of alkylbenzyldimethylammonium chlorides homologues, have an important biocide character. According to the usage field of Benzalkonium chloride, its concentrations are variable and it is an irritant substance when it is used in the high concentration. Particularly useful for protecting the baby's skin condition can cause discomfort, such as dermatitis. Due to these adverse effects, amount of Benzalkonium chloride has to be examined in wet wipes. In this study, a quantitative analysis method of Benzalkonium chloride was developed by using capillary electrophoresis that would become a powerful separation technique. As capillary electrophoresis was applied on different solutions of Benzalkonium chloride successfully, the homologues of Benzalkonium chloride were identified and confirmed in this study. Quantitative analysis was carried out by the external standard method. The electrophoretic separation was performed by using 75 mM pH 6.0 phosphate buffer solution with 30 percent Acetonitrile as electrolyte. The separation voltage was 10 kV, and the temperature was held at 18 centigrade degree. Samples which include 5 mg/ml Benzalkonium chloride solutions were introduced by hydrodynamic way by using 50.0 mbar pressure during 3 s. The method developed was validated according to ICH guideline and performed on wet wipes that contains 0.1 percent the concentration of Benzalkonium chloride, without any extraction productively and the calibration curves showed good linearities.

Keywords: Benzalkonium chloride, wet wipe, capillary electrophoresis, analysis, quantitative.

ÖZET

Günümüzde süregelen hızlı yaşam koşulları nedeni ile temizlik-hijyen konusunda anlık ve hızlı çözümler getiren ürünlerin kullanımı artmıştır. Bu ürünlerin başında da el temizliği için kullanılan ıslak mendiller gelmektedir. Islak mendiller bebek temizliğinden yüzey temizliğine dek birçok değişik amaç için üretilmektedir. El temizliği için kullanılan ıslak mendillerin antibakteriyel tipleri de vardır. Benzalkonyum klorür, benzetonyum klorür, setrimid, klorheksidin, hekzaklorofen, alkol, hidrojen peroksit, hekzamin hipurat, triklosan, setilpiridinyum klorür, dekualinyum klorür ıslak mendillerde antibakteriyel ajan olarak kullanılırlar. Benzalkonyum klorür sağlık ve kozmetik preparatlarında yaygın olarak kullanılan kuaterner amonyum surfaktandır ve topikal antiseptik ve medikal cihaz dezenfektanı olarak da kullanılır. Alkilbenzildimetilamonyum klorür homologlarının karışımı olan Benzalkonyum klorür önemli biyosit özelliğe sahiptir. Benzalkonyum klorür kullanım alanına göre farklı konsantrasyonlarda bulunur ve yüksek konsantrasyonlarda kullanıldığında irritan bir maddedir. Koruyucu olarak kullanıldığı durumda da özellikle bebek cildinde dermatit gibi rahatsızlıklara sebep verebilir. Tüm bu nedenlerden dolayı yer aldığı ürünlerde Benzalkonyum klorür derişimi izlenmelidir. Bu çalışmada, güçlü bir ayırma tekniği olan kapiler elektroforez kullanılarak bir Benzalkonyum klorür kantitatif analiz metodu geliştirilmiştir. Çalışmada Benzalkonyum klorür' ün farklı solüsyonlarında kapiler elektroforez başarılı bir şekilde uygulandığı gibi, Benzalkonyum klorür homologları tanmlanmış ve doğrulanmıştır. Kantitatif analiz, dış standart metodu ile uygulanmıştır. Elektroforetik ayırma elektrolit olarak yüzde 30' u Asetonitril 75 mM pH 6.0 fosfat tamponu solüsyonu kullanılarak yapılmıştır. Ayırma voltajı 10 kV, ve sıcaklık 18 santigrat derece' dir. 5 mg/ml derişiminde Benzalkonyum klorür solüsyonu içeren numuneler 3 saniye boyunca 50 mbar basınç uygulayarak hidrodinamik yol ile uygulanmıştır. Geliştirilen metot ICH klavuzuna göre valide edilip, yüzde 0.1 konsantrasyonda Benzalkonyum klorür solüsyonu içeren ıslak mendiller üzerinden ekstraksiyon yapmadan verimli olarak test edilmiştir ve kalibrasyon eğrisi iyi doğrusallık göstermiştir.

Anahtar kelimeler: Benzalkonyum klorür, ıslak mendil, kapiler elektroforez, analiz, kantitatif.

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LIST OF ABBREVIATIONS

A **:** Eddy-diffusion parameter, related to channeling through a non-ideal packing [m]

ACN **:** Acetonitrile

B **:** Diffusion coefficient of the eluting particles in the longitudinal direction, resulting in dispersion [m^2 s^{-1}]

BAC **:** Benzalkonium chloride

BGE **:** Background electrolyte

BP **:** British Pharmacopeia

C **:** Resistance to mass transfer coefficient of the analyte between mobile and stationary phase [s]

CE **:** Capillary electrophoresis

CGE **:** Capillary gel electrophoresis

CITP **:** Capillary isotachophoresis

CIEF **:** Capillary isoelectric focusing

CMC **:** Critical micelle concentration

CZE **:** Capillary zone electrophoresis

DNA **:** Deoxyribonucleic acid

E **:** The electric field strength

EP **:** European Pharmacopeia

F **:** The friction coefficient

FE **:** The force imparted by electrical field

Ff **:** Frictional force

GC **:** Gas chromatography

HETP **:** Height equivalent to a theoretical plate, a measure of the resolving power of the column

HPLC **:** High performance liquid chromatography

LC **:** Liquid chromatography

LOD **:** Limit of detection

LOQ **:** Limit of quantification

m **:** Slope

MECC : Micellar electrokinetic capillary chromatography

Min : Minute

pKa : Dissociatian constant

p.s.i : per square inch

q : Effective charge

r : The hydrodynamic radius

RSD : Relative standard deviation

RP : Reversed phase

s : Second

SDBS : Sodium dodecylbenzenesulphonate

sub-μM : Submicrometer

sub-pM : Subpycnometer

u : Linear Velocity [m s^{-1}]

USP : United States Pharmacopeia

UV : Ultraviolet

v/v : volume/volume

$\mathbf{V_{ep}}$: The velocity of the ion

x : The separation axis

μ_{ep} : Electrophoretic mobility

μ_{eo} : The electroosmotic mobility

μ_{app} : The apparent mobility

η : The viscosity

υ_{eo} : The electroosmotic velocity

ε : Dielectric constant

ζ : The zeta potential

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1. INTRODUCTION

Wet wipes can serve a number of personal and household purposes. Although marketed primarily for wiping infants' backsides in diaper changing, it is not uncommon for consumers to also use the product to clean floors, toilet seats, and other surfaces around the home. Also, there are antibacterial types of wet wipes for hand cleaning. The most used antibacterial agent used in wet wipes having antibacterial property is benzalkonium chloride (BAC).

BAC is used in a variety of purpose. According to the usage field of BAC, its concentrations are variable. If it is used as a preservative-stabilizer, the range of concentration has to be 0.007-0.01%; and it has to be 0.01-0.2% for the usage of instead of disinfectant [1].

On the other hand, although BAC is an irritant substance, it has an advantage of preventing microbial infection such as bacteria, fungi and protozoa. The other advantage of BAC is one of the most often used preservatives because of its efficiency, its stability and its low cost. However, the disadvantage of BAC is that several toxic effects have been obtained. BAC prevent microbial proliferation and therefore must be used at effective concentrations, although toxic. Indeed, microbial proliferation and its consequences could be worse than the toxicity of the preservative. As a result, microbial proliferation and its consequences could be worse than the toxicity of the preservative [2].

The aim of this work was to investigate the concentration of BAC by using the method of capillary electrophoresis (CE) in order to develop the suitable research analysis and analyze BAC which is used as an active substance in antibacterial wet wipe samples.

2. BAC

BAC is synthetized by the nucleophilic reaction of alkyldimethylamine with benzylchloride [3]. The appearance of BAC is white or yellowish-white powder or gelatinous, yellowish-white fragments and hygroscopic. On heating it forms a clear molten mass. It is very soluble in water and in ethanol (96%). An aqueous solution froths copiously when shaken [4].

BAC can have different alkyl chain lengths ranging from C_8H_{17} to $C_{18}H_{37}$ with different chain lengths conferring different chemical properties. The percentage of the alkyl chain distribution can vary as the compound is derived from natural sources, such as coconut oil and tallow [5].

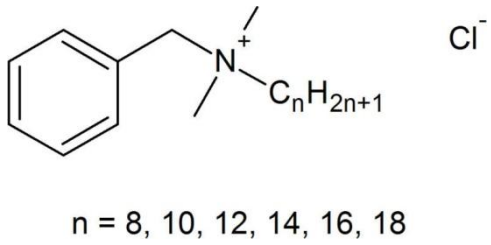

Figure 2.1. The molecular structure of BAC

BAC belongs to the alkyldimethylbenzylammonium type of compounds. BAC forms normal micelles, and is a common surfactant used as a foaming and cleansing agent, conditioner and bactericide. It is also used in ophthalmic medication and contact lens solutions, hand washes, antimicrobial soaps, as well as in skin wound cleaners and preoperative skin preparation solutions [6].

pKa_a value of BAC is 5.7. In this case, BAC is cationic under pH 5.7 and non-ionic over it [7].

Also, BAC is a major preservative component in eyedrops used to prevent bacterial contamination in multi-dose bottles during the treatment period of eye diseases. Such bactericidal agents are necessary for patient safety because the multi-use containers for eyedrops often lead to improper use [8].

In commercial preparations the aliphatic alkyl chains possess lengths of 12, 14 and 16 carbon atoms and at low concentrations BAC occurs in aqueous solutions as positively loaded, boundary surface active ions with amphipathic conformation [9].

3. BENZALKONIUM CHLORIDE DETERMINATION TECHNIQUES

BAC is used for different purposes and has different effects on user or target objects. Thus its concentration should be monitored. A number of methods were developed for determination of BAC. These methods were presented in the following section according to the technique used.

3.1. Titrimetric Methods

In the United States Pharmacopeia (USP), assay of BAC as raw material was performed by titrimetric method as following extraction of BAC with chloroform from sample [10].

According to USP, assay for solutions including BAC, was performed by titrimetric method as following. A quantity of BAC solution is evaporated or diluted with water. The assay method for BAC solution is continued as above method for BAC [10].

A quantity of BAC is weighed, transferred to a glass and water and chloroform are added. An amount of freshly prepared potassium iodide solution is added, the stopper is insert in the separator, it is shaken, and the layers are allowed to separate, and the chloroform layer is discarded. The aqueous layer is washed with chloroform, and discarded the washings. The aqueous layer is transferred to conical flask, and the separator is rinsed with water, adding the washings to the flask. Cold hydrochloric acid is added to the flask, mixed, and titrated with 0.05 M potassium iodate until the solution becomes light brown in color. Chloroform is added, inserted the stopper into the flask, and shaken. Also a blank determination is performed, using amount of water as the sample [10].

In the European Pharmacopeia (EP), BAC assay was done by titrimetric method as following extraction of BAC by using methylene chloride [4].

A quantity of BAC is dissolved in water. Amount of the solution is transferred to a separating funnel, and methylene chloride is added. Then 0.1 M sodium hydroxide and freshly prepared solution of potassium iodide are added. The methylene chloride layer is allowed to separate and discarded. Hydrochloride acid is added to the aqueous layer,

allowed to cool and titrated with 0.05 M potassium iodide until the deep-brown colour is almost discharged. Methylene chloride is added and continued the titration shaking until the methylene chloride layer no longer changes color. Blank titration is carried out [4].

In a study, a potentiometric titration method was used in concentrations of 10^{-7} – 10^{-1} M of BAC. By using this method, BAC was determined in pharmaceutical products as well as in pure solutions by using a new modified carbon paste electrodes [11].

3.2. Spectroscopic Methods

In 1998 Kovac and et al declared a spectrophotometric method by using an ion-pair extraction method in order to determine BAC [12]. An ion-pair is formed between the quaternary ammonium salt and an anionic dye such as bromthymol blue, bromphenol blue, methyl orange, etc. At a specific pH, the ion-pair is extracted into an organic solvent which is immiscible with water. In addition to the traditional batch procedures, flow injection analysis methods are also frequently used with spectrophotometric, indirect atomic absorption and ion-selective electrode detectors. In Kovács-Hadady' s study which is determination of BAC in eye-drops, at pH 4.40 and 9.62, the working curve is linear in the 1.98 x 10^{-6} to 2.40 x 10^{-5} M concentration range; however the sensitivity drops to about one third in basic solutions [12].

In 1998 Bernal and et al studied for nasal drugs which include BAC by UV-spectroscopic multicomponent method [13].

3.3. Chromatographic Techniques

3.3.1. Liquid Chromatography Methods

Liquid chromatography (LC) can successfully separate each BAC homologue allowing them to be determined respectively. However, when BAC is extracted from treated wood with organic solvent, co-extracted components in the sample solution might interfere with analysis using LC with ultraviolet (UV) detection. In Miyauchi' s study which is a quantitative determination method of BAC in treated wood, peak results were obtained at 262 nm. The temperature was 40°C and the mobile phase was ACN - 100 mM ammonium formate buffer, pH 3.5 (70:30 v/v). Interference by extractives of five wood species on quantitative determination of BAC by using LC-UV

analysis method was confirmed, except with western hemlock, particularly at a spiking level of 1 mg/g [14].

In J.L. Bernal's study, nasal drugs which include BAC were studied by HPLC [13].

3.3.2. Gas Chromatography Methods

The most of Gas Chromatography (GC) methods include on based on a modified Hofmann degradation of BAC with subsequent analysis of the formed benzyldimethylamine and the corresponding alkane. Another technique involves chemical derivatization of the BAC to introduce specific functional groups into the derivatives which are amenable to GC with enhanced detecability using electron capture and nitrogen-specific thermionic detecting systems [15].

In Cybulski' s study [15], new and simple method for the analysis of BAC was presented. A GC had been used to pyrolyze BAC in a specific and reproducible manner to yield two tertiary amines for each homologue of BAC present. These were separated by GC and were used to determine the homologue composition of the BAC. These determinations could be made with an analysis time of 25 min/sample. Measurements were carried out using a GC fitted with a flame ionization detector. The carrier gas for the GC was nitrogen. Samples of BAC for analysis were prepared as solutions in methanol (0.5%) and standards [15].

3.3.3. High Performance Liquid Chromatography Methods

High performance liquid chromatography (HPLC) method was used to determine BAC homologues in various pharmaceutical preparations [16, 17, 19].

For example, in Dudkiewicz-Wilczyńska's study [16], an HPLC method was developed for BAC determination in aerosol preparations. The method was simple and did not require particular sample preparation for the tests. They were characterized by high selectivity and high measurement precision. The performed validation confirmed the usefulness of the method. In order to optimize the conditions of BAC separation, occurring in the sample along with other substances, a number of experiments were performed in which the influence of the solid phase and the mobile phase, and the column temperature, were measured. The following separation conditions were

selected: mobile phase ACN - 0.075 M acetate buffer, pH 5.0, in the proportion 55:45 (v/v), respectively, in an isocratic system. Flow rate was 1 ml/min., and the column temperature -25 °C. The detection was performed at 262 nm wave length, and the injection volume was 50 µl as a standard, 0.1 mg/ml BAC water solution was used. The developed method allows fast assessment of BAC identity. Validation of the HPLC method was discussed in the study. Linearity, repeatability and accuracy were assessed. Researchers claimed that this method could be used for assessment of identity and content of individual BAC homologues in various pharmaceutical preparations [16].

In Kümmerer' s study [17], BAC was extracted from the sewage and injected into an HPLC system. In HPLC system, the oven temperature was set at 15 °C for the analysis of BAC and the mobile phase was chloroform-methanol (80:20, v/v). The method was applied to highly complex effluent samples from different sized European hospitals. The method for the extraction analysis of BAC in sewage proved to be highly reproducible and effective [17].

In Hou's study [18], analyses were performed by on HPLC system connected to an UV-visible detector operating at 200 nm for the determination of BAC in ophthalmic solutions. The study was done for determination of BAC in ophthalmic solutions. Isocratic elution was performed with a mixture of 60% acetonitrile (ACN) and 0.1 M sodium acetate adjusted to pH 5. The quantitation of BAC was carried out using the external standard method to construct calibration curve covering the range 5-50 µg/ml [18].

Also, in a nasal drug solution formulation, the application of gradient HPLC for the determination of the preservative BAC was tricky due to column fouling and lack of selectivity [19].

3.3.4. Capillary Electrophoretic Methods

In Hou' s study [18], CE experiments were performed on a P/ACE MDQ system equipped with a UV-Vis detector for the determination of BAC in ophthalmic solutions. Separations were carried out in a untreated fused-silica capillary and effective length of 50 cm. The UV detector was operated at 200 nm. All electrophoresis runs were performed at temperature 20 °C. The on-column detection window was made by burning a small section of the external polymide coating and scraping of the burned

residue with methanol. The most effective separation conditions was 40 mM phosphate buffer with 40% ACN at pH 4.0 and the sample injection of up to 10 s at 2 p.s.i, and and an applied voltage + 15 kV at the injection end of the capillary. The calibration curves were linear with coefficient of determination $R^2 > 0.99$ in the range of 1.0 – 20 µg/ml [18].

In Para' s study [20], the electrophoretic data was carried out using uncoated fused-silica capillaries and a 50 mM acetic acid-ammonium acetate buffer solution at pH 4.5 containing %80 ACN as carrier electrolyte. The separation was performed by applying a voltage of + 20 kV and the temperature was held at 25 °C. Direct detection was performed at 215 nm. Most of the publications that analyze BAC by CE generally use nonvolatile salts such as phosphate buffers as carrier electrolytes or sulfonic electrolytes. Quantitative analysis was carried out by the internal standard method and the calibration curves showed good linearities (R^2<0.98). The CE–ESI-MS/MS method was successfully applied to the analysis of BAC in different ophtalmic solutions. LOD value was 0.5 for C_{12} peak, 0.6 for C_{14} peak, 0.5 for C_{16} peak and 0.5 for C_{18} peak [20].

Another CE method was developed for analysis of BAC by Jimidar [19]. The analysis was carried out on CE system at constant voltage of 15 kV. The zones were detected with a UV detector at 215 nm. Sample injection was performed by applying 35 mbar positive pressure for 10 s. BAC was in concentrations of 0.15 or 0.125 mg/ml. The described method was suitable for the determination of BAC in the formulation of alniditan which is a anti-migraine drug and is therefore applied to test the quality of different alniditan formulations in stability and release studies [19,30].

In 1996 Altria and at al [21] investigated the homologues of BAC by CE. A 37 cm long, 75 µm capillary filled with 25 mM NaH_2PO_4 solution was used for separation. The capillary was rinsed between injections; initially for 1 min with 0.1 M NaOH, followed by 1 min with the electrolyte. Sample was introduced by pressure application for 5 s. The separation voltage was + 15 kV with detection at 200 nm and an operating temperature of 30 °C. Sample solutions were prepared in an internal standard solution consisting of imidazole (0.1 mg/ml) dissolved in water. For raw material testing 0.1 mg/ml BAC concentrations were used. Analysis was performed on Beckman P/ACE 5000 CE instruments. The CE method is capable of performing a variety of testing using a relatively simple electrolyte and a standard, inexpensive capillary. Use of the CE method in preference to methods specified in the British Pharmacopeia (BP)

monograph, or HPLC alternatives, can have significant benefits in terms of analysis time, costs, improved quality and solvent purchase/disposal. The CE method is applicable to both assay of BAC which can result in significant savings, compared to HPLC, in terms of analysis time and the cost of consumables and reductions in solvent disposal and purchase. The method has significant advantages over current BP-specified testing and HPLC alternatives. Satisfactory validation data was achieved for sensitivity, linearity, precision recovery, robustness and selectivity [21].

3.4. Capillary Electrophoresis Technique

CE is the most efficient separation technique available for the analysis of both large and small molecules. Modern CE was generated by the production of narrow-bore capillaries and the development of highly sensitive on-line chromatographic detection methods. This analytical method is a combination of GC and HPLC techniques [22].

Electrophoresis has been applied to a variety of difficult analytical separation problems: inorganic anions and cations, amino acids, catecholamins, drugs, vitamins, carbohydrates, peptides, proteins, nucleic acids, nucleotides, polynucleotides, and numerous other species. A superior strength of electrophoresis is its unique ability to separate charged macromolecules. For many years, electrophoresis has been the powerhouse method of separating proteins and nucleic acids, for which it offers unparalleled resolution [23].

Also, CE is the most rapidly expanding separation technique in pharmaceutical analysis and is a rival to HPLC in its general applicability. The instrumentation is quite straightforward, apart from the high voltages required, but the parameters involved in optimizing the technique to produce separation are more complex than those involved in HPLC. CE is preferred to HPLC, where highly selective separation is required [24].

The ability to obtain high separation efficiencies by the application of a voltage across a capillary was invented in early 1980s. The late 1990s, a variety of the separation mechanisms have seen in CE and instrumentation developments aimed at addressing practitioner's needs [25].

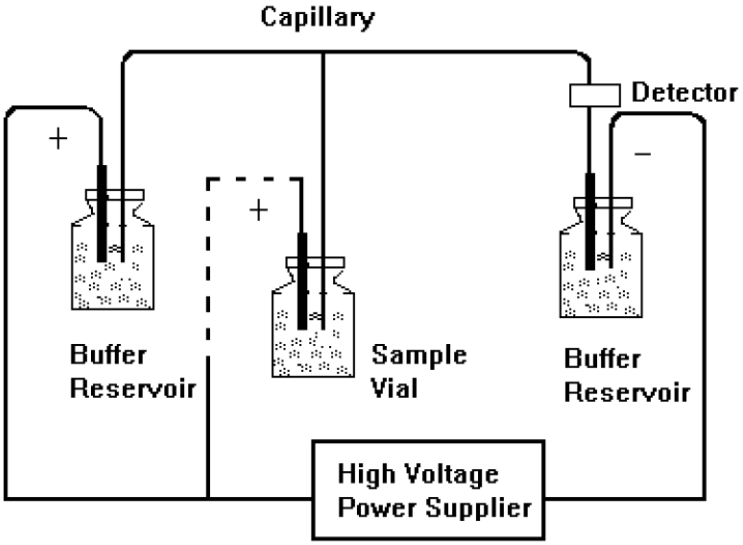

Figure 3.4.1. The instrumental set-up of a CE system

The basic instrumental set-up, which is illustrated in Figure 3.4.1., consists of a high voltage power supply , a fused silica capillary, two buffer reservoirs, two electrodes, and an on-column detector. Sample injection is accomplished by temporarily replacing one of the buffer reservoirs with a sample vial. A specific amount of sample is introduced by controlling either the injection voltage or the injection pressure [22].

The unprecedented resolution of CE is a result of the technique's extremely high efficiency. The separation efficiency of CE and other high-resolution techniques such as chromatography and field-flow fractionation is modeled by the Van Deemter Equation [22].

The Van Deemter equation relates the resolving power of a chromatographic column to the various flow and kinetic parameters which cause peak broadening, as follows:

$$\text{HETP} = A + \frac{B}{u} + C.u \qquad \text{Equation 3.4.1.}$$

In open tubular capillaries, A will be zero as the lack of packing means channeling does not occur. In packed columns, however, multiple distinct routes ("channels") exist through the column packing, which results in band spreading. In the latter case, A will not be zero.

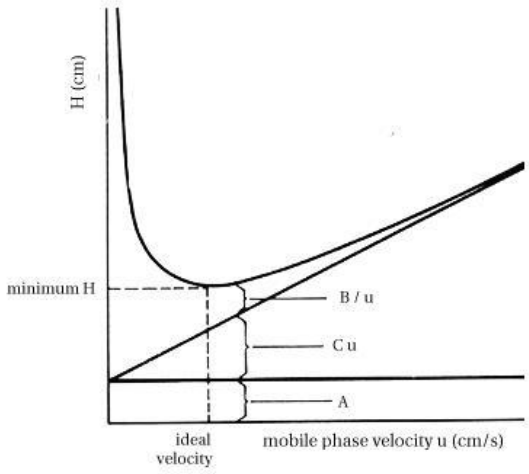

Figure 3.4.2. The van Deemter Graphic

The form of the van Deemter equation is such that HETP achieves a minimum value at a particular flow velocity. At this flow rate, the resolving power of the column is maximized, although in practice, the elution time is likely to be impractical. Differentiating the van Deemter equation with respect to velocity, setting the resulting expression equal to zero, and solving for the optimum velocity yields the following cells [26].

$$u = \sqrt{\frac{B}{C}}$$ Equation 3.4.2.

CE analyses are usually very fast, use little sample and reagents, and cost much less than chromatography or conventional electrophoresis. Although modern CE is still in its teenage years, it has demonstrated tremendous potential for a wide range of applications, from small molecules that include inorganic ions etc., even living cells [22].

CE has proved to be a new analytical tool suitable for the investigation of both seized preparations of illicit drugs, as well as biological samples. This technique displays several characteristics, such as electrophoretic and/or chromatography-like separation mechanisms, negligible consumption of samples and reagents, instrumental simplicity

and multiple detection modes, which make it unique on the scene of modern analytical technology. The possibility of interfacing with mass spectrometry is an additional feature of CE crucial in the forensic environment, which is ready to become commercially available, mainly with electrospray or atmospheric pressure ionization interfaces. Therefore, CE seems to have an important future in forensic toxicology as well as in other fields of CE crucial in the forensic science and is already ready for use as a complement to the usual, more consolidated analytical techniques [27] .

CE is continuing to expand its application within the area of pharmaceutical analysis. Much of this advance is due to its simplicity and low cost of operation. Often simple buffer solutions can be used to separate simultaneously a range of different substances. For example, a simple low-pH phosphate buffer has been validated to quantify a range of basic drugs and excipients. CE has been used to quantify levels of anionic detergents such as sodium dodecylbenzenesulphonate (SDBS) and is capable of revolving SDBS homologues [21].

Also, LC and CE are the techniques most frequently used for the analysis of the cationic compounds, allowing the separation and determination of the most important BAC homologues. RP LC with UV-detection has been used for the determination of BAC in ophthalmic solutions, nasal sprays and some biological samples such as blood and tissues. Fluorescence detection has also been used for the analysis of some hospital effluents. Other detection systems such as conductometric detection have also been used but only with solutions. The cationic characteristics of BAC make CE a useful technique for the separation and determination of BAC homologues in drug formulations, in nasal and ophthalmic solutions and in some studies using standards. In all cases, the concentration is high enough and the detection limits of the CE-UV methods do not present a problem [20].

3.4.1. Electrophoresis

Electrophoresis is defined as the migration of ions under the influence of an electric field. The force (FE = qE) imparted by the electrical field is proportional to its effective charge, q, and the electric field strength, E. The translational movement of the ion is opposed by a retarding frictional force ($Ff = fv_{ep}$), which is proportional to the

velocity of the ion, v_{ep}, and the friction coefficient, f. The ion almost instantly reaches a steady state velocity where the accelerating force equals the frictional force [22].

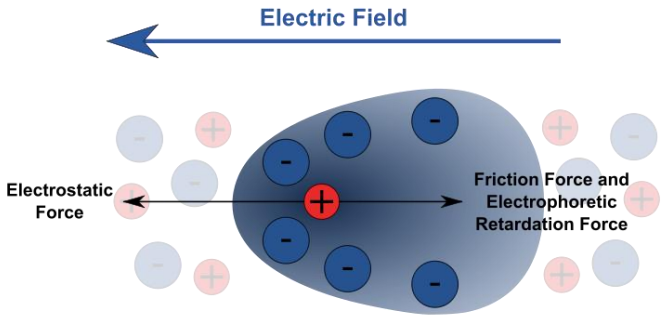

Figure 3.4.1.1. Illustration of electrophoresis

$$qE = f\,v_{ep} \qquad \text{Equation 3.4.1.1.}$$

Rearranging Equation 3.4.1.1. yields

$$v_{ep} = \frac{q}{f} E = \mu_{eo} E \qquad \text{Equation 3.4.1.2.}$$

Here μ_{ep} is the electrophoretic mobility of the ion, which is a constant of proportionality between the velocity of the ion and the electric field strength. The electrophoretic mobility is proportional to the charge of the ion and inversely proportional to the friction coefficient [22].

The friction coefficient (f) of the moving ion is related to the hydrodynamic radius, r of the ion and the viscosity, η, of the surrounding medium,

$$f = 6\pi\eta r$$ Equation 3.4.1.3.

so, a larger hydrodynamic radius translates to a lower electrophoretic mobility [22].

3.4.2. Electroosmosis

Electroosmosis refers to the movement of the buffer in the capillary under the influence of the electric field. The inner surface of a fused silica capillary is covered with silanol groups (Si-OH), which are ionized to SiO– at pH > 2. The negatively charged surface is counterbalanced by positive ions from the buffer, forming the so-called electric double layer. Under the influence of the electric field, the positive ions in the diffuse part of the double layer migrate towards the cathode; in doing so they entrain the waters of hydration, which results in electroosmotic flow. The Equations of electroosmotic flow are identical to those developed for electrophoresis, as both phenomena are complementary. The electroosmotic velocity, υ_{eo} is defined by

$$\upsilon_{eo} = \mu_{eo} E$$ Equation 3.4.2.1.

where μ_{eo} is the electroosmotic mobility, a constant of proportionality between the electroosmotic velocity and the electric field strength. Electroosmotic mobility is proportional to the dielectric constant, ε, of the medium and the zeta potential, ζ, at the capillary–buffer interface, and inversely proportional to the viscosity, η, of the medium [22].

$$\mu_{eo} = \frac{\varepsilon\zeta}{\pi\eta}$$ Equation 3.4.2.2.

The zeta potential is largely dependent on the electrostatic nature of the capillary surface, and to a small extent, on the ionic nature of the buffer. In fused silica capillaries, electroosmosis is diminished at low pH because protons convert the charged

SiO surface to SiOH, causing a decrease in the zeta potential. Electroosmosis also decreases with increasing ionic strength, due to collapse of the double layer. Electroosmotic flow can be reduced by coating the capillary with a material that suppresses ionization of the silanol groups, such as polyacrylamide or methylcellulose [22].

3.4.3. Apparent mobility

The apparent mobility, μ_{app}, of a solute is a vector sum of the electrophoretic mobility, μ_{ep}, of the solute plus the electroosmotic mobility, μ_{eo}, of the solution [22].

$$\mu_{app} = \mu_{ep} + \mu_{eo} \qquad \text{Equation 3.4.3.1}$$

The apparent velocity, υ_{app} , of a solute is directly proportional to μ_{app} and the electric field strength, E, across the capillary [22],

$$\upsilon_{app} = \mu_{app} E \qquad \text{Equation 3.4.3.2.}$$

Neutral solutes migrate in the same direction and velocity as the electroosmotic flow and are not separated. Cations and anions are separated based on differences in their apparent mobilities. For cations, which move in the same direction as the electroosmotic flow, μ_{ep} and μ_{eo} have the same sign, so $\mu_{app} > \mu_{ep}$. The electrophoresis of anions, on the other hand, is in the opposite direction of electroosmosis, so for anions μ_{ep} and μ_{eo} have opposite signs. At moderate pH values (pH > 3), electroosmotic flow is generally higher than electrophoretic flow causing anions to migrate towards the cathode, which is where the detector is typically located. At lower pH, electroosmosis is weak and anions may never reach the detector unless the polarity of the instrument is reversed in order to change the location of the detector from the cathode end to the anode end of the capillary [22].

3.4.4. Detection Methods

Most of HPLC detection methods with some modifications to CE may apply. Different detectors can be used in CE devices such as UV spectrophotometer, fluorescence detector or mass spectrometry detector. The most used detector is the UV-Vis detector or DAD detector [22].

Optical detector usage makes the usage of CE limited due to pathway of electromagnetic radiation. Pathway of the light is depend on the capillary internal diameter. Because the light affects the materials analyzed only through this distance which is changes 20- 100 μm [22].

It makes the LOD (Limit of detection) determination is limited due to the cross-section of the capillary the CE (Figure 3.4.4.1) [28].

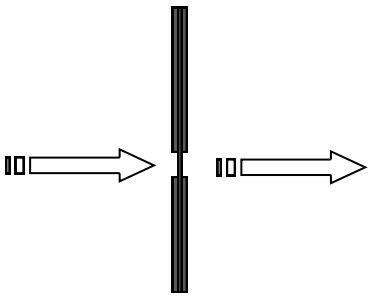

Figure 3.4.4.1. Representation of the determination of the beam path with capillary diameter when spectroscopic methods used

To achieve 3- 10 fold increase in minimum sensitivity, extended optical beam path cells can be used (Figure 3.4.4.2) [28].

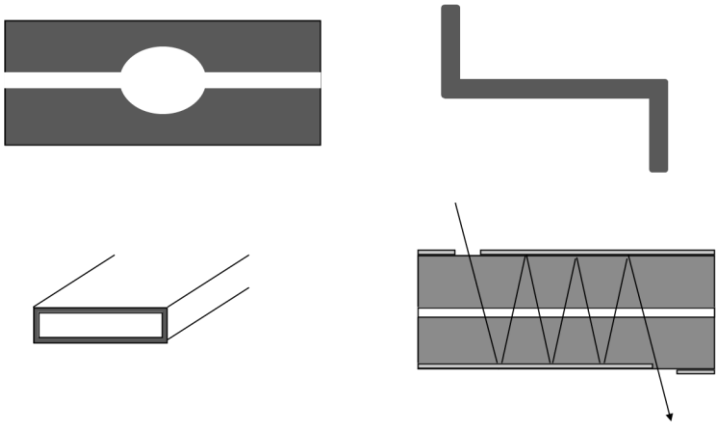

Figure 3.4.4.2. Cell designs are developed to improve LOD value

LODs at sub-μM and sub-pM levels are typical with amperometric detection and laser-induced fluorescence detection, respectively [22].

The linear dynamic range of CE applications has been as narrow as one order of magnitude or as wide as six orders of magnitude, depending on the analyte. Reproducibility is typically in the range of 1-2% for peak area and 3-7% for peak height [22].

3.4.5. Separation Methods

The main separation modes used in CE are capillary zone electrophoresis (CZE), micellar electrokinetic capillary chromatography (MECC), capillary isotachophoresis (CITP), capillary gel electrophoresis (CGE), and capillary isoelectric focusing (CIEF). Each of these modes use a high voltage to achieve highly efficient separations.

3.4.5.1. Capillary Zone Electrophoresis

CZE is the simplest form of CE. In this mode sample is applied as a narrow zone (band), which is surrounded by the separation buffer. As an electric field is applied, each component in the sample zone migrates according to its own apparent mobility. Ideally, all sample components will eventually separate from each other to form individual zones of pure material. However, neutral molecules cannot be separated because they migrate at the velocity of electroosmotic flow. The separation of

charged molecules is accomplished most efficiently when differences among the apparent velocities of the components are maximized and random dispersion of the individual zones is minimized.

3.4.5.2. Micellar Electrokinetic Capillary Chromatograph

The development of MECC has extended CE applications to the separation of both neutral and charged molecules through the use of micelles in the separation buffer. Micelles are aggregates of amphiphilic monomers known as surfactants, which possess a hydrophilic head and a hydrophobic tail. The hydrophobic tail can be a straight or branched chain of hydrocarbon, or a steroidal skeleton; the hydrophilic head can be either cationic, anionic, zwitterionic or nonionic. When surfactant molecules exceed their critical micelle concentration (CMC), they are pushed together by the polar medium. In aqueous solutions, spherical micelles form with the hydrophobic tails pointing inward and the hydrophilic heads facing outward. In MECC, micelles serve as a pseudo-stationary phase that resembles the stationary phase in reverse-phase HPLC. Various types of interaction can occur between solutes and micelles, including hydrophobic, electrostatic and hydrogen-bonding interactions. The partitioning of solutes between micelles and the aqueous buffer can be controlled by the concentration and chemical composition of the surfactant and other additives to the buffer, such as organic solvents, ionic salts, chiral selectors, ion-pairing and complexing agents. Partitioning can also be manipulated by changes in temperature, pH, and ionic strength. Separation in MECC is a result of the combined effect of the differential partitioning of molecules between the aqueous buffer and the micellar phase, as well as any differential migration of ionic species.

3.4.5.3. Capillary Isotachophoresis

CITP is isotachophoresis performed in a capillary. In CITP, a sample is inserted between a leading electrolyte and a trailing electrolyte without electroosmotic flow. The leading electrolyte has a higher mobility and the trailing electrolyte has a lower mobility than ions in the sample zone. CITP cannot be used to separate cations and anions at the same time. Detection methods are based on conductivity, differential conductivity, or direct UV adsorption. In the latter case, spacers are placed between

analyte bands. The spacers contain solutes that do not absorb in the UV and have mobilities between those of the two neighboring bands.

3.4.5.4. Capillary Gel Electrophoresis

CGE is an adaptation of traditional slab gel electrophoresis to the capillary format; it is CZE performed in a polymeric gel medium. CGE is potentially useful for the separation of large biological molecules such as proteins and DNAs, which have similar electrophoretic migration rates in free solution due to their similar charge-to-mass ratios. CGE separates molecules according to their size in an nonconvective medium. Separation media include non-crosslinked polymers such as linear polyacrylamide, polyethylene glycol and cellulose derivatives, as well as crosslinked polymers or gels, such as polyacrylamide and agarose. The entangled polymer network inside the capillary serves as a molecular sieve in which smaller molecules migrate faster than large. The polymer network reduces the solute diffusion rate and the adsorption of solute to the capillary wall, while suppressing electroosmotic flow. These features increase efficiency, which permits the use of a shorter column. With crosslinked polymers, the resolution of the capillary can be easily optimized for a given range of molecular weights by varying the total monomer concentration and degree of cross-linking. However, non-crosslinked polymers can be easily flushed out of the capillary when a problem develops and can be reloaded to generate a fresh capillary for each separation.

3.4.5.5. Capillary Isoelectric Focusing

CIEF is used for the separation of amphoteric substances such as proteins, peptides, amino acids, and pharmaceuticals in polymer matrices as well as free solutions. Separation in CIEF is based on differences in the isoelectric point of sample components rather than differences in apparent velocity.

A series of zwitterions are used to generate a pH gradient inside the focusing capillary. Ampholytes that are positively charged migrate towards the cathodewhile those that are negatively charged migrate towards the anode. Therefore, the pH increases at the cathode side of the capillary and decreases at the anode side. When an ampholyte reaches its own pI and is no longer charged, its migration ceases.

As a result, a stable pH gradient is formed. If the analyte has a net positive charge, it migrates towards the cathode. During its migration, it eventually encounters a pH at which it has a zero net charge and ceases to migrate. The pH gradient is smoother when a larger number of ampholytes are used. In order to prevent the migrations of buffers from the buffer reservoirs into the capillary, the pH of the electrolyte in the cathode must be higher than the pIs of all the basic ampholytes and the pH of the buffer in the anode must be lower than the pIs of all the acidic ampholytes. CIEF is most effective when electroosmotic flow and other convective forces are eliminated or greatly suppressed. However, it is still possible to perform CIEF in the presence of electroosmosis, as long as the electroosmotic velocity of the solution inside the capillary does not exceed the electrophoretic velocities of the analytes. CIEF is a true focusing technique. If a solute molecule from a focused band happens to diffuse away from the zone center, it immediately loses or gains protons, and thus acquires charge. In its charged state, the solute migrates back toward the zone center.

Eventually, a steady state is reached where the zones are stationary and sharply focused.

3.4.6. Validation of an Analytical Method [29]

The discussion of the validation of analytical procedures is directed to the four most common types of analytical procedures; identification tests, quantitative tests for impurities' content, limit tests for the control of impurities, quantitative tests of the active moiety in samples of drug substance or drug product or other selected component(s) in the drug product.

Although there are many other analytical procedures, such as dissolution testing for drug products or particle size determination for drug substance, these have not been addressed in the initial text on validation of analytical procedures.

A brief description of the types of tests is provided below.

Identification tests are intended to ensure the identity of an analyte in a sample. This is normally achieved by comparison of a property of the sample (e.g., spectrum, chromatographic behavior, chemical reactivity, etc) to that of a reference standard; Testing for impurities can be either a quantitative test or a limit test for the impurity in a sample. Either test is intended to accurately reflect the purity characteristics of the

sample. Different validation characteristics are required for a quantitative test than for a limit test; Assay procedures are intended to measure the analyte present in a given sample. In the context of this document, the assay represents a quantitative measurement of the major component(s) in the drug substance. For the drug product, similar validation characteristics also apply when assaying for the active or other selected component(s). The same validation characteristics may also apply to assays associated with other analytical procedures (e.g., dissolution).

The objective of the analytical procedure should be clearly understood since this will govern the validation characteristics which need to be evaluated. Typical validation characteristics which should be considered are listed below:

Table 3.4.6.1. Typical validation characteristics

Parameters
Accuracy
Precision
Repeatability
Intermediate Precision
Specificity
Detection Limit
Quantitation Limit
Linearity
Range

The table lists those validation characteristics regarded as the most important for the validation of different types of analytical procedures. This list should be considered typical for the analytical procedures cited but occasional exceptions should be dealt with on a case-by-case basis. It should be noted that robustness is not listed in the table but should be considered at an appropriate stage in the development of the analytical procedure.

Furthermore revalidation may be necessary in the following circumstances; changes in the synthesis of the drug substance, changes in the composition of the finished product, changes in the analytical procedure.

The degree of revalidation required depends on the nature of the changes. Certain other changes may require validation as well.

3.4.6.1. Analytical Procedure

The analytical procedure refers to the way of performing the analysis. It should describe in detail the steps necessary to perform each analytical test. This may include but is not limited to: the sample, the reference standard and the reagents preparations, use of the apparatus, generation of the calibration curve, use of the formulae for the calculation, etc.

3.4.6.2. Specifity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.

Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedure(s).

3.4.6.3. Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

3.4.6.4. Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

Precision should be investigated using homogeneous, authentic samples. However, if it is not possible to obtain a homogeneous sample it may be investigated using artificially prepared samples or a sample solution.

The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

3.4.6.4.1. Repeatability

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.

3.4.6.4.2. Intermediate precision

Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc.

3.4.6.4.3. Reproducibility

Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology).

3.4.6.5. Detection Limit

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

3.4.6.6. Quantitation Limit

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

3.4.6.7. Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

3.4.6.8. Range

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

3.4.6.9. Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

4. EXPERIMENTAL

4.1. Reagents

BAC, ACN, methanol, disodium hydrogen phosphate and sodium dihydrogen phosphate were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

Wet wipe samples containing BAC and wet wipe solutions containing BAC were kindly supplied from Kardeşler Uçan Yağlar Ltd. Şti.

4.2. Standard Solutions and Background Electrolyte (BGE)

Stock solution of BAC was prepared by dissolving of BAC in methanol as 5 mg/mL. Standard solutions for analysis studies were prepared by diluting stock solution of BAC with methanol.

Firstly phosphate buffer and acetate buffer were used as electrolyte. Phosphate buffer was selected as electrolyte. Ionic strength of phosphate buffer is more effective than the others as electrolyte. Also, Methanol and ACN were studied as organic solvent and ACN was selected as organic solvent. For determination of ACN ratio, a study was performed at six different values at 10%, 20%, 30%, 40%, 50%, 60% and ACN ratio was selected as 30%. Background electrolyte was a mixture of phosphate buffer (75 mM and pH 6.0) and acetonitril (70:30).

Sample solutions were used diluting directly and not by using a preparation step.

4.2.1. Preparing of Sample Solutions

Five wet wipes were wringed. 100 µL of sample solutions were diluted by adding 1400 µL methanol. Although the concentration of the solution is matters not the amount, wringing was made by the same tecnique by the same person.

4.2.2. Preparing of Wetting Solutions

Wetting solution was also analyzed. For this purpose 100 µL of wetting solution containing 0.1% BAC was diluted with 1400 µL methanol. This solution was used

directly for measurement. This study was designed to determine compare with wet wipe samples.

4.2.3. Preparing of Dry Wipe Adsorption Capacity Solution

This study was designed to determine how much that BAC adsorption in wet wipe. To determine the amount of adsorbed BAC on dry wet wipe, wetting solution containing 0.1% BAC was treated on dry wipe samples in ratio of 2.80 (w/w) (used value to moisture the dry wet wipes in commercial products) and allowed to stand at room temperature for a week. Then five wet wipes were wringed and 100 µL of obtained solution is diluted by adding 1400 µL methanol. The dry wipe adsorption capacity solution was used directly for measurement.

4.3. Apparatus

CE separation technique was applied on an Agilent 3D Capillary Electrophoresis system equipped with a DAD detector. Separation was achieved on an uncoated fused silica capillary which was 750 µm I.D. and 47 cm in lenght. The wavelength was selected as 214.2 nm, due to the maximum absorption in spectrums obtained by the DAD detector of the CE system. The capillary was termostatted at 18 °C during all experiments. The typical electrophoregram of BAC standard are presented in Figure 4.3.1.

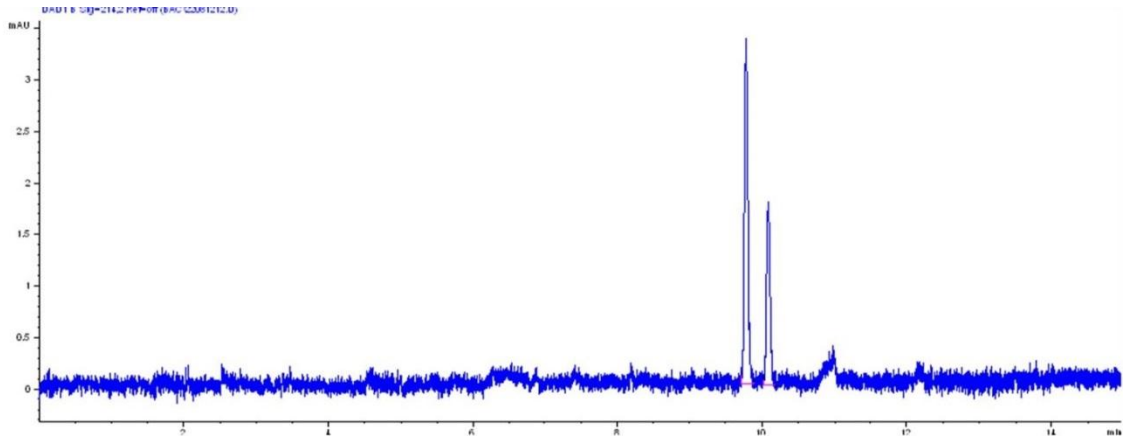

Figure 4.3.1. The typical electropherogram of BAC standard at 214.2 nm

Ultrapure water (18.2 MΩ.cm at 25°C) was obtained by using Millipore Simplicity UV apparatus.

pH Measurements were made with a digital pH meter under stirring conditions at room temperature (25.0±0.1 °C).

4.4. Washing Programme

A washing programme was applied for new capillaries through the following steps: a) flush capillaries with 1.0 M NaOH (20 min), b) flush with 0.1 M NaOH (20 min) and c) flush with water (20 min). Between each measurement, the capillary was conditioned by flushing with 1.0 M NaOH (1.5 min), 0.1 M NaOH (1.5 min), water (3 min), BGE (5 min) respectively. After the conditioning step, samples were injected hydrodynamically by using 50 mbar pressure for 3 s at the anodic end of the capillary and 10 kV separation voltage was applied. At the end of the measurement the capillary was rinsed with water for 3 min.

4.5. Optimization of Electrolyte Concentration

For optimization of electrolyte concentration, a study was performed for six different electrolyte concentration at 25 mM, 50 mM, 75 mM, 100 mM, 200 mM and 300 mM. Peak area values and peak shapes were investigated and optimum concentration value was selected.

4.6. Optimization of pH Value

To evaluate the effect of pH, different electrolyte solutions were investigated having different pH value at 5, 6, 7 and 8. Peak area values and peak shapes were investigated and optimum pH value was selected.

4.7. Optimization of Temperature

A study was aimed to optimize temperature. Therefore, the study was performed for four different temperatures at 18ºC, 20ºC, 24ºC and 28ºC. Peak area values, peak shapes, repeatability of peaks were investigated and optimum temperature value was selected.

4.8. Effect of Acetonitrile Ratio

For optimization of ACN ratio, a study was applied for six different ACN ratio at 10%, 20%, 30%, 40%, 50% and 60%. Peak area values and peak shapes were investigated and optimum ACN ratio was selected.

4.9. Optimization of Separation Potential

A study was performed how to effect of potential variable, for five different values at 5 kV, 8 kV, 10 kV, 15 kV and 20 kV. Repeatability of peaks and peak shapes were investigated and optimum separation potential was selected.

4.10. Calculations of Limit of Detection and Limit of Quantification

In this study, LOD and LOQ values were calculated according to USP. Signal to noise rates (3s/m for LOD, 10s/m for LOQ) were used for this purpose. First of all blank signals were recorded from electrophoregrams and then standard deviation of these values were calculated. Obtained value was used to determine LOD and LOQ values. Related calculations were as following:

5. RESULTS AND DISCUSSION

5.1. Optimization of Electrolyte Concentration

According to certificates of analysis, C_{12} and C_{14} peaks are present in standard. The first peak is C_{12}. Because C_{12} is more small molecule and it moves fast.

For optimization of electrolyte concentration, a study was performed for six different electrolyte concentration at 25 mM, 50 mM, 75 mM, 100 mM, 200 mM and 300 mM. Electrophoregrams obtained for this study are presented in Figure 5.1.1.

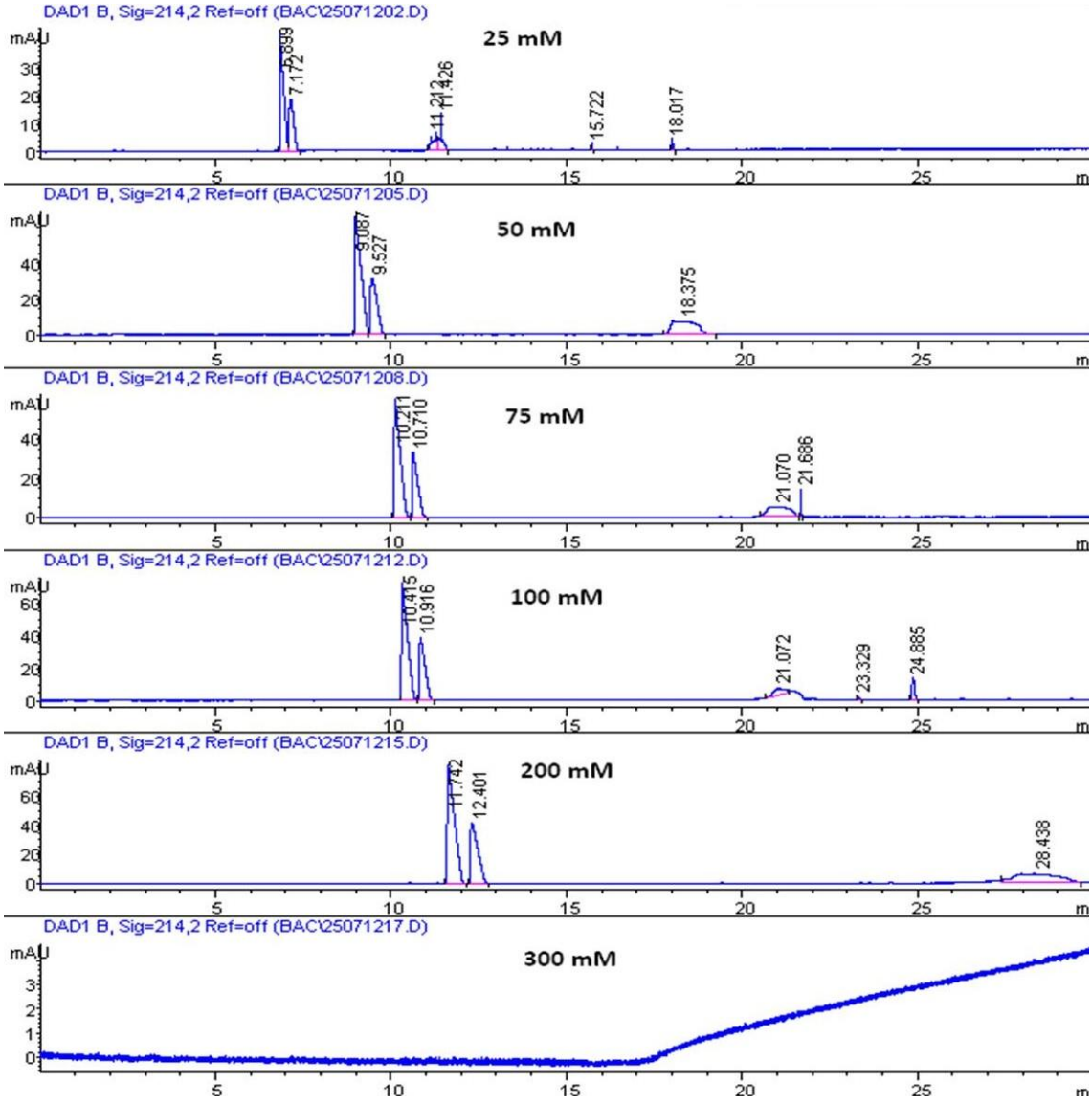

Figure 5.1.1. The electropherograms obtained by using pH 6.0 phosphate buffer solution with 30% ACN, applied 10 kV at 18°C for optimization of electrolyte concentration

The electrophoretic separation was performed by using pH 6.0 phosphate buffer solution with 30% ACN as electrolyte. The separation voltage was 10 kV, and the temperature was held at 18°C.

Effect of the electrolyte concentration on obtained peak area values are also shown in Table 5.1. and in Figure 5.1.2. and 5.1.3. at a graph.

Table 5.1.1. Effect of the electrolyte concentration in the range of 25 – 300 mM on obtained peak area values

Electrolyte Concentration (mM)	C_{12} Peak Area	C_{14} Peak Area	Resolution Value
25	256.4	158.8	0.0158
50	393.63	159.97	0.0178
75	428.63	213.6	0.0250
100	419.2	213.5	0.0208
200	387.5	152.6	0.0208
300	0	0	-

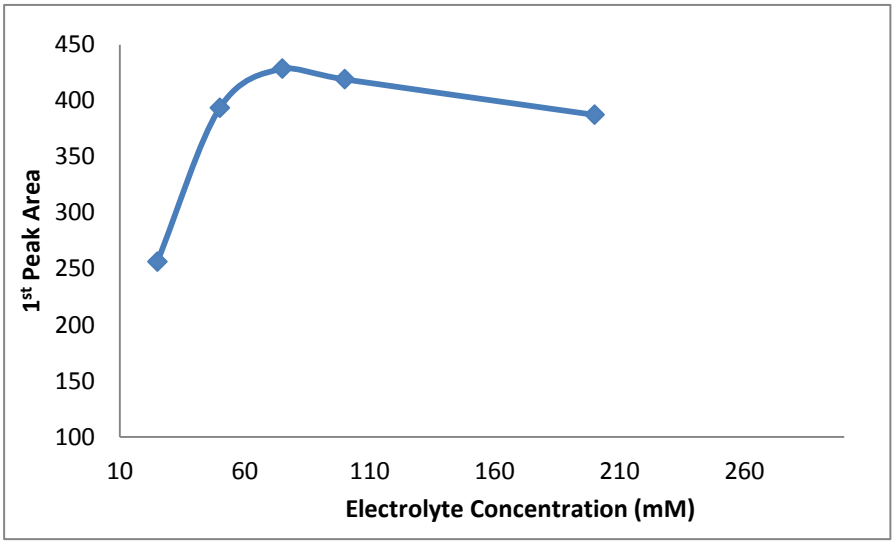

Figure 5.1.2. Effect of the electrolyte concentration in the range of 25 – 300 mM on obtained C_{12} peak area values

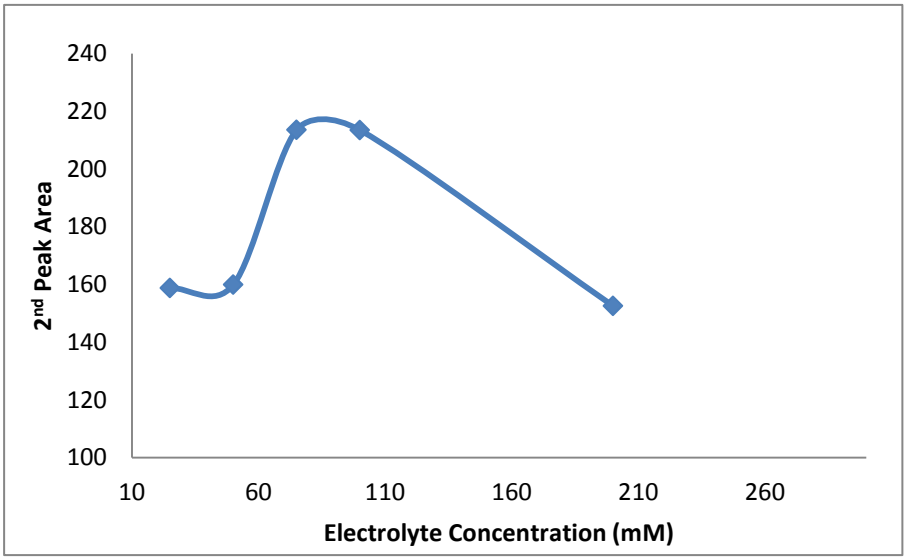

Figure 5.1.3. Effect of the electrolyte concentration in the range of 25 – 300 mM on obtained C_{14} peak area values

In this study, 75 mM electrolyte concentration was selected due to peak shapes and peak area values. This value was used for following studies.

5.2. Optimization of pH Value

To evaluate the effect of pH, different electrolyte solutions were investigated having different pH value as 5, 6, 7 and 8. The electrophoregrams obtained are presented in Figure 5.2.1.

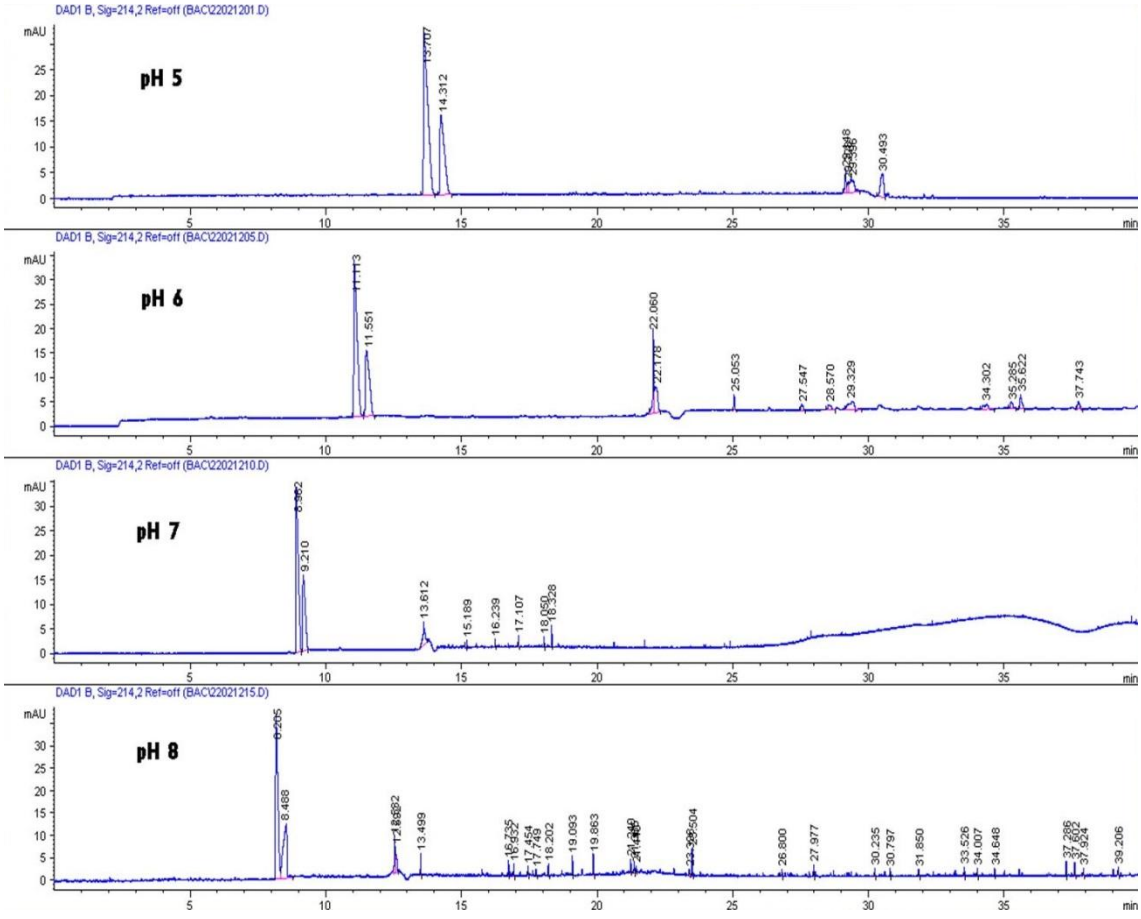

Figure 5.2.1. The electropherogram that obtained by using 75 mM phosphate buffer phosphate buffer solution with 30% ACN, applied 10 kV at 18°C for optimization of pH

The electrophoretic separation was performed by using 75 mM phosphate buffer solution with 30% ACN as electrolyte. The separation voltage was 10 kV, and the temperature was held at 18 °C.

Effect of the pH value on obtained peak area values are also shown in Table 5.4. and in Figure 5.2.1. and 5.2.2. as a graph.

Table 5.2.1. Effect of the pH in the range of 5.0 – 8.0 on obtained peak area values

pH	C_{12} Peak Area	C_{14} Peak Area	Resolution Value
5	239.15	118.2	0.0350
6	252.47	131.55	0.0229
7	218.02	116.23	0.0350
8	198.46	110.6	0.0273

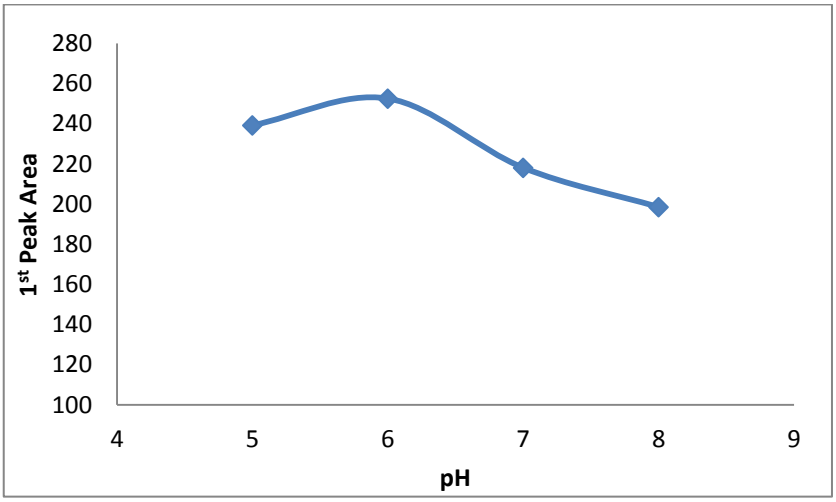

Figure 5.2.2. Effect of the pH in the range of 5.0 – 8.0 on obtained C_{12} peak area values

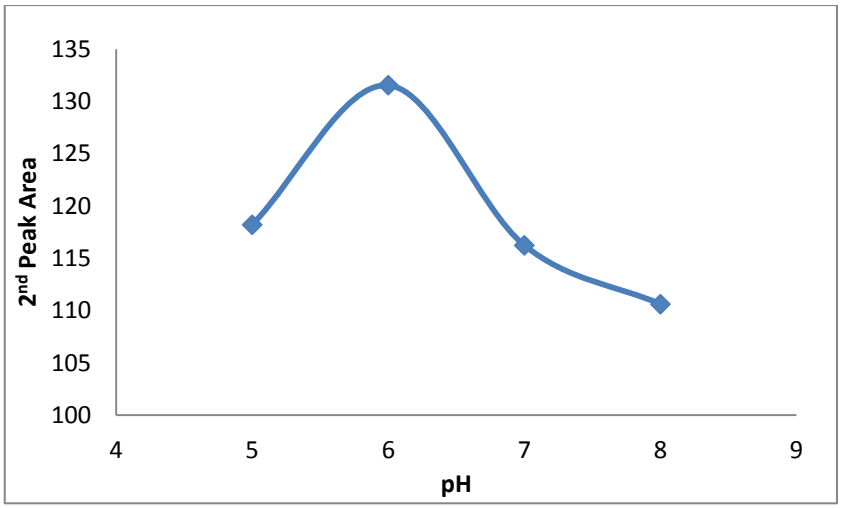

Figure 5.2.3. Effect of the pH in the range of 5.0 – 8.0 on obtained C_{14} peak area values

In this study, optimum pH value was selected as pH 6 due to peak shapes and peak area values. This value was used for following studies.

5.3. Optimization of Temperature

For optimization of temperature, a study was performed at four different points as 18ºC, 20 ºC, 24ºC and 28ºC. The electrophoregrams obtained are presented in Figure 5.3.1.

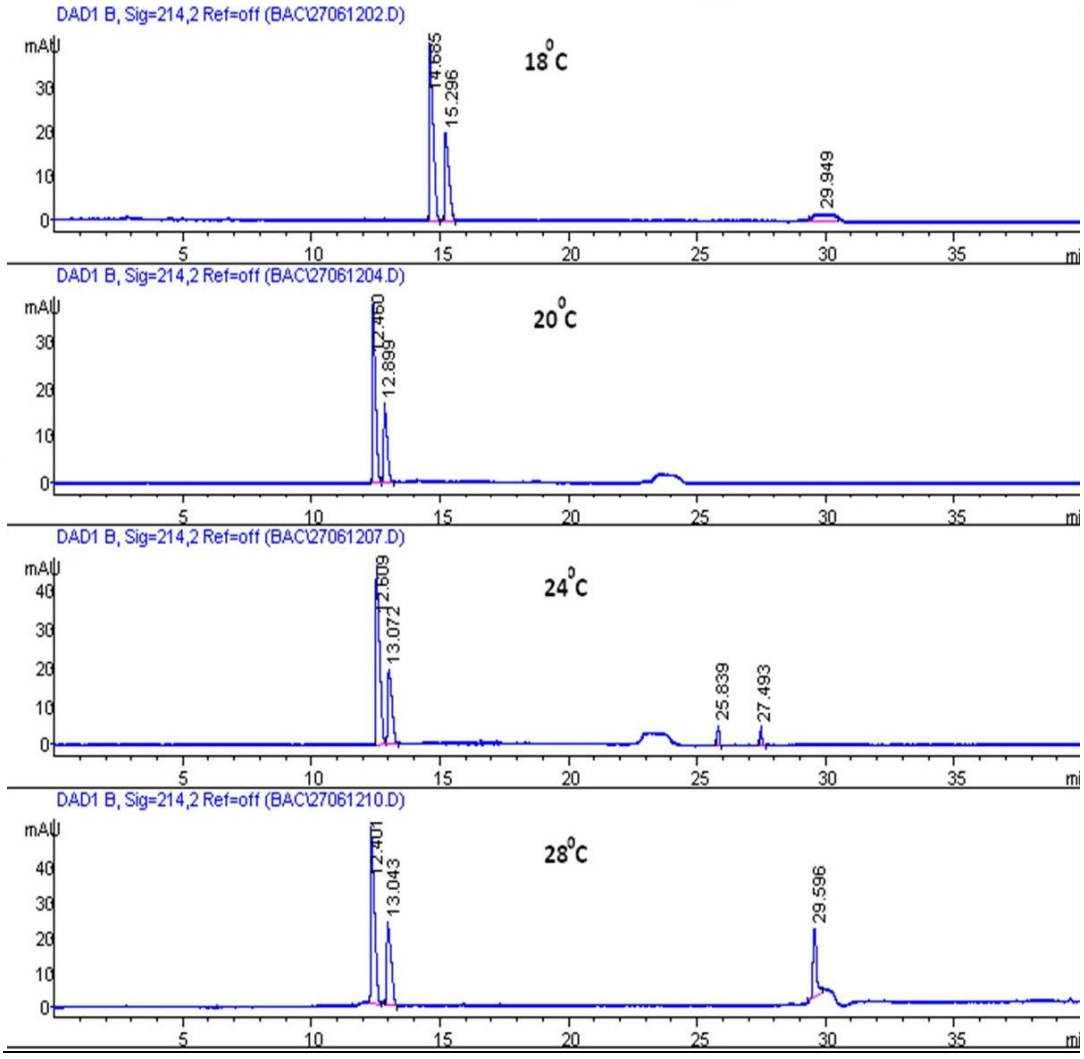

Figure 5.3.1. The electropherogram that obtained by using 75 mM pH 6.0 phosphate buffer phosphate buffer solution with 30% ACN, applied 10 kV for optimization of temperature

The electrophoretic separation was performed by using 75 mM pH 6.0 phosphate buffer solution with 30% ACN as electrolyte. The separation voltage was 10 kV.

Effect of temperature on obtained peak area values are also shown in Table 5.3. and in Figure 5.3.2. and 5.3.3. as a graph .

Table 5.3.1. Effect of the temperature in the range of 18 – 28°C on obtained peak area values

Temperature (°C)	C_{12} Peak Area	C_{14} Peak Area	Resolution Value
18	417.9	212.7	0.0350
20	332.9	168.55	0.0250
24	357.0	180.25	0.0368
28	1022.4	458.86	0.0267

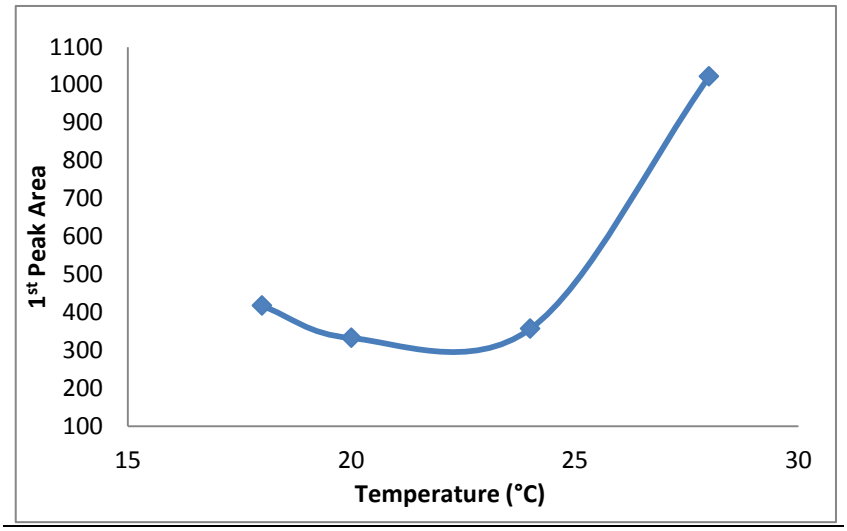

Figure 5.3.2. Effect of the temperature in the range of 18 – 28°C on obtained C_{12} peak area values

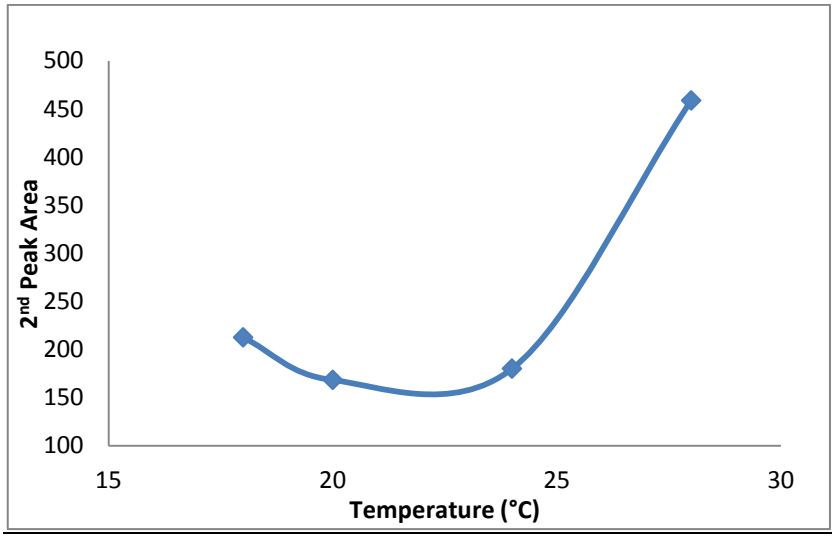

Figure 5.3.3. Effect of the temperature in the range of 18 – 28°C on obtained C_{14} peak area

In this study, 18°C temperature was selected due to peak shapes and repeatability of peaks. This value was used for following studies.

5.4. Effect of Acetonitrile Ratio

For optimization of ACN ratio, a study was performed at six different values at 10%, 20%, 30%, 40%, 50% and 60%. The electrophoregrams obtained for this study are presented in Figure 5.4.1.

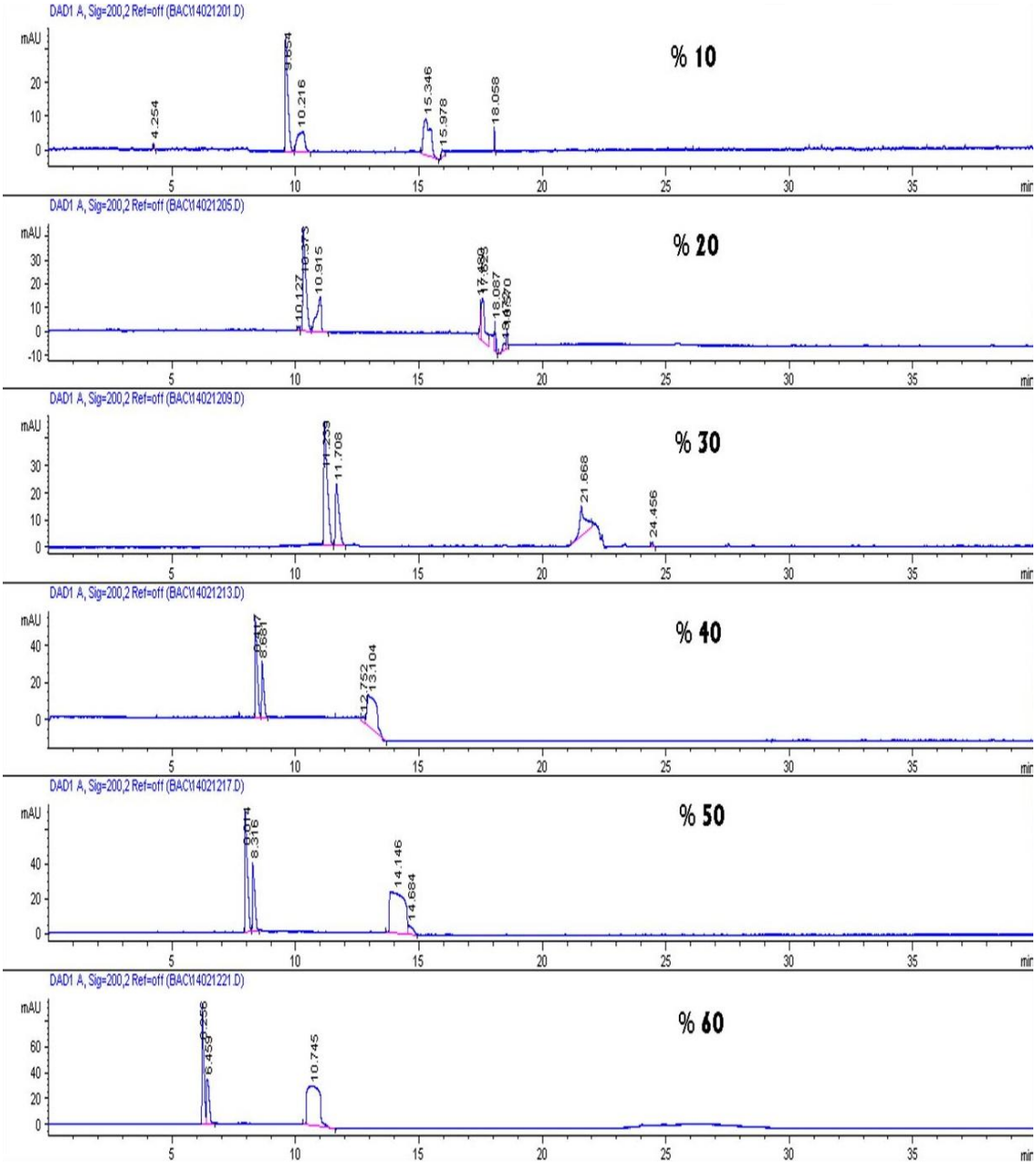

Figure 5.4.1. The electropherogram that obtained by using 75 mM pH 6.0 phosphate buffer phosphate buffer solution, applied 10 kV at 18°C for effect of ACN ratio

The electrophoretic separation was performed by using 75 mM pH 6.0 phosphate buffer solution with ACN as electrolyte. The separation voltage was 10 kV, and the temperature was held at 18°C.

Effect of the ACN ratio on obtained peak area values are also shown in Table 5.4. and in Figure 5.4.2. and 5.4.3. as a graph .

Table 5.4.1. Effect of the ACN ratio in the range of 10 – 60% on obtained peak area values

Ratio of ACN (%)	C_{12} Peak Area	C_{14} Peak Area	Resolution Value
10	336.4	1593.83	0.0216
20	398.3	204.97	0.0400
30	421.55	216.22	0.0286
40	400.3	205.3	0.0500
50	488.35	254.29	0.0800
60	453.96	244.56	0.0200

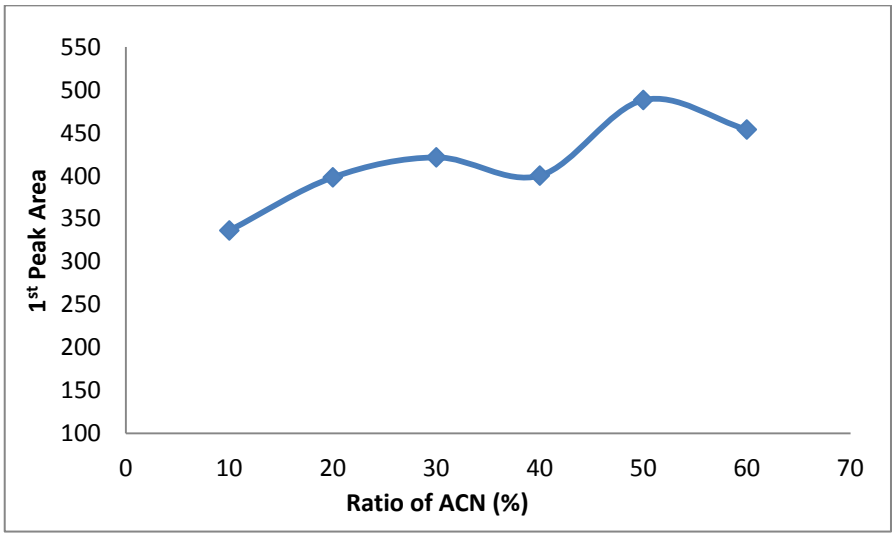

Figure 5.4.2. Effect of the ACN ratio in the range of 10 – 60% on obtained C_{12} peak area values

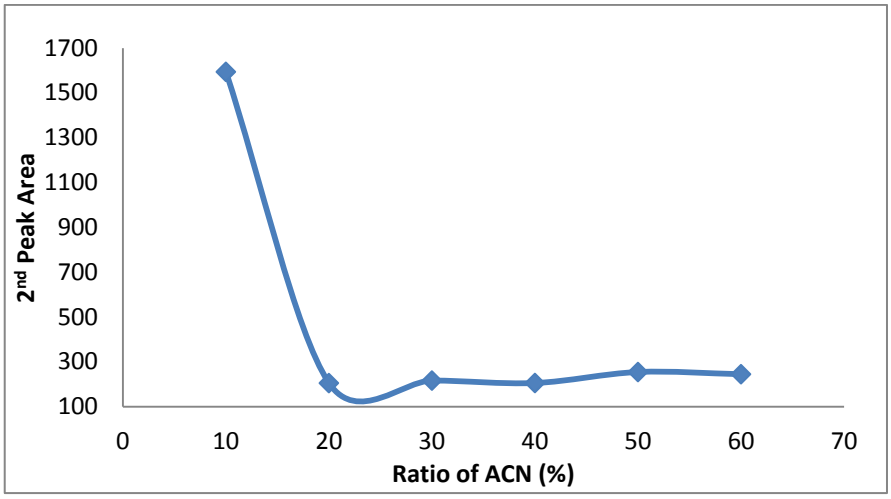

Figure 5.4.3. Effect of the ACN ratio in the range of 10 – 60% on obtained C_{14} peak area values

In this study, optimum ACN ratio was selected as 30% due to peak shapes and peak area values. This value was used for following studies.

5.5. Optimization of Separation Potential

For optimization of potential, a study was performed for five different values at 5 kV, 8 kV, 10 kV, 15 kV and 20 kV. The electrophoregrams obtained are presented in Figure 5.5.1.

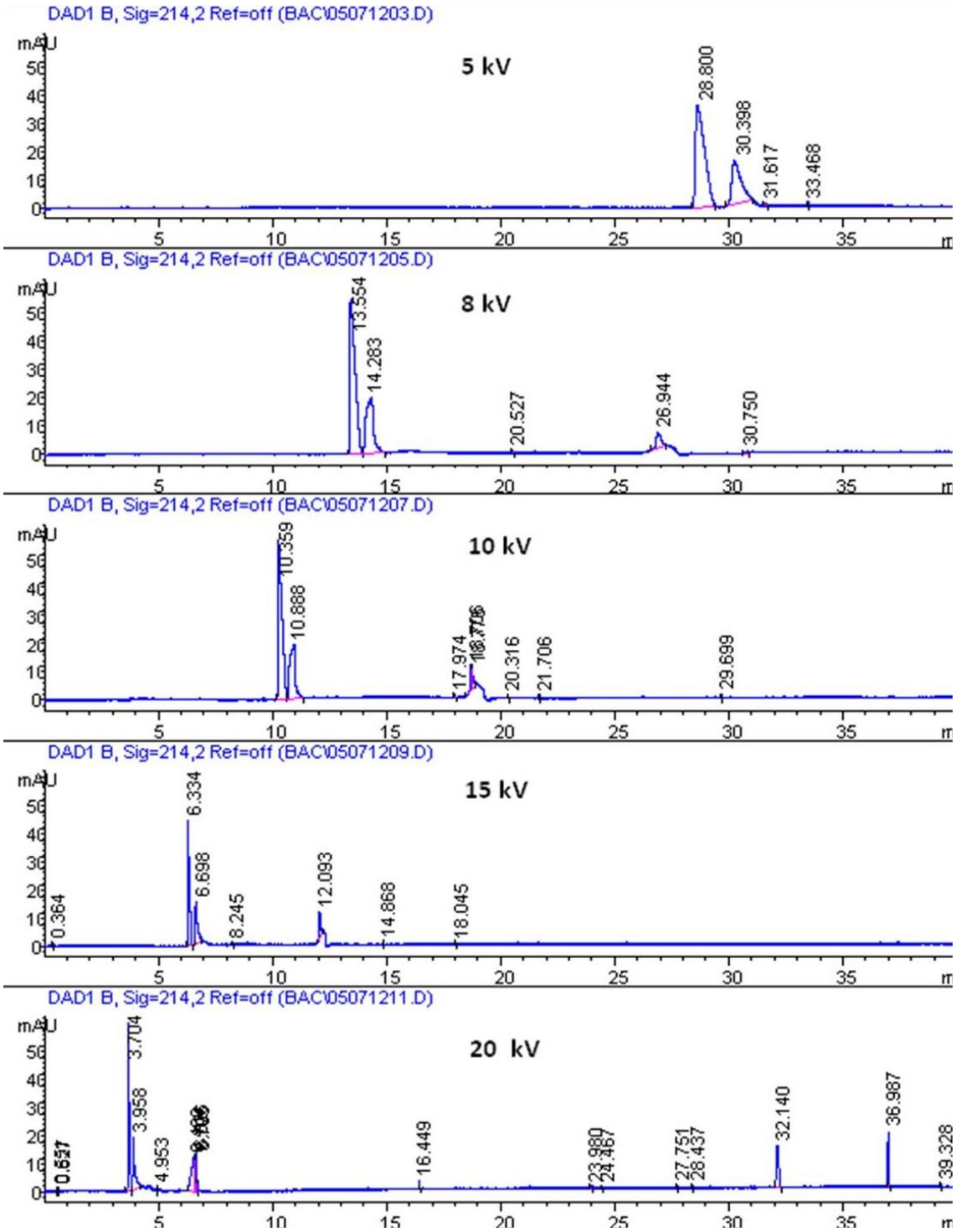

Figure 5.5.1. The electropherogram that obtained by using 75 mM pH 6.0 phosphate buffer phosphate buffer solution with 30 % ACN at 18°C for optimization of separation potential

The electrophoretic separation was performed by using 75 mM pH 6.0 phosphate buffer solution with 30% ACN as electrolyte. The temperature was held at 18°C.

Effect of separation potential on obtained peak area values are also shown in Table 5.5. and in Figure 5.5.2. and 5.5.3. as a graph.

Table 5.5.1. Effect of the separation potential in the range of 5.0 – 8.0 on obtained peak area values

Separation Potential (kV)	**C_{12} Peak Area**	**C_{14} Peak Area**	**Resolution Value**
5	1238.9	646.7	0.0356
8	858.5	454.3	0.0360
10	641.25	315.8	0.0421
15	309.85	145.95	0.0270
20	257.75	113.75	0.0050

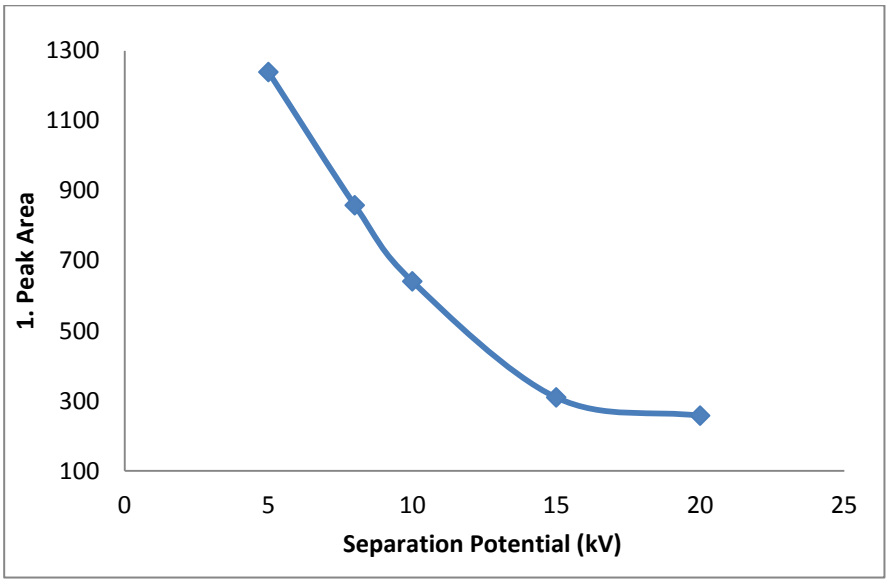

Figure 5.5.2. Effect of the separation potential in the range of 5.0 – 8.0 on obtained C_{12} Peak area values

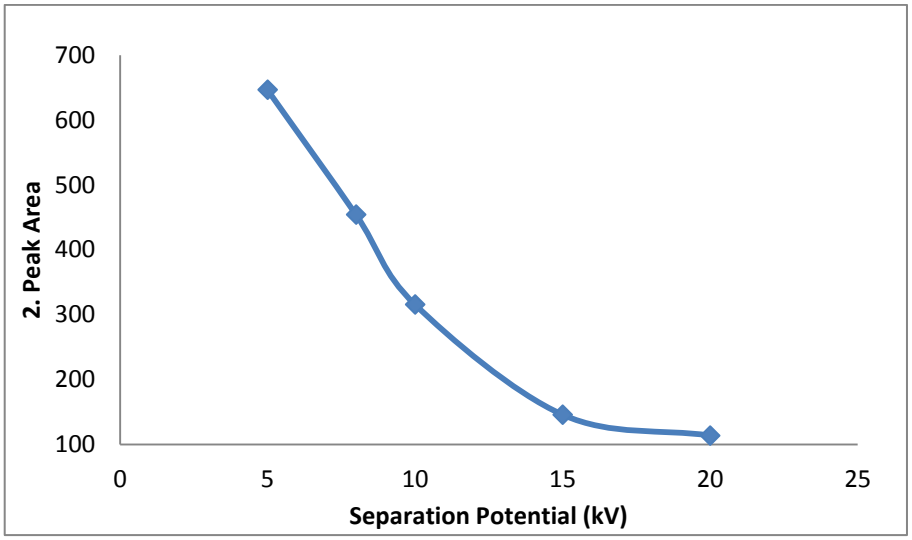

Figure 5.5.3. Effect of the separation potential in the range of 5.0 – 8.0 on obtained C_{14} Peak area values

Although more peak area values were obtained at lower separation potential, peak shapes and repeatability of peaks were better using 10 kV than using 5 kV or 8 kV according to repeated studies. Thus 10 kV potential value was selected as separation potential.

5.6. Specificity Studies

The specificity of method was assessed by analyzing placebo solution, electroforegram were compared for any interference from the matrix or any of the assay reagents. The electroforegram is presented in Figure 5.6.1.

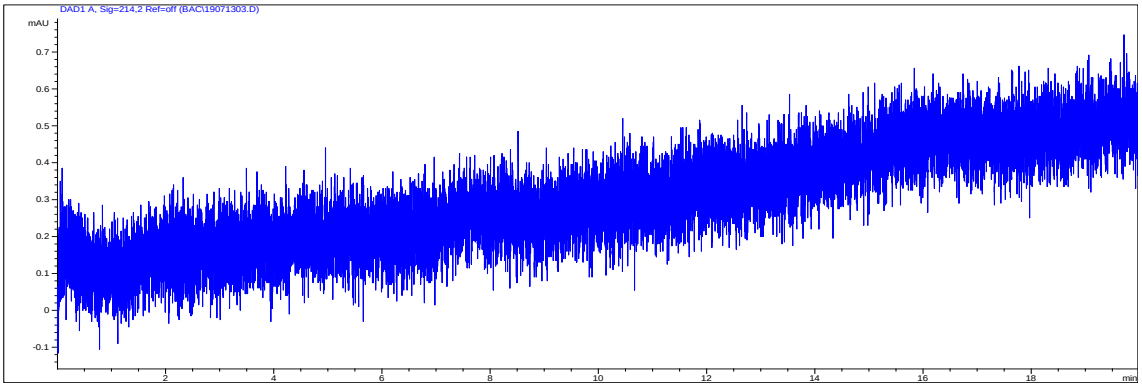

Figure 5.6.1. The placebo sample electropherogram obtained by using pH 6.0 phosphate buffer solution with 30% ACN, applied 10 kV at 18°C for optimization of electrolyte concentration

The electropherogram is clear and there are no peaks in the electropherogram.

5.7. Recovery Studies

The recovery studies were applied for accuary. Recoveries of BAC in wet wipe for C_{12} peak and C_{14} peak are presented in Table 5.8.1.

Table 5.7.1. Calculated recovery and relative standard deviation values for known concentration of BAC at different concentrations

Concentration (M)	Intraday				Interday			
	C_{12} Peak		C_{14} Peak		C_{12} Peak		C_{14} Peak	
	Recovery %	RSD %	Recovery %	RSD %	Recovery %	RSD %	Recovery %	RSD %
0.0075	119.34	3.158	121.25	4.345	75.24	27.968	68.19	24.22
0.06	89.81	0.520	88.67	0.742	96.8	16.866	96.76	15.26
0.2	105.91	1.202	107.89	4.444	99.9	17.518	101.66	16.70

The method was found more succesfull at low concentration.

5.8. Precision Studies

The results of the interday and intraday tests of BAC are listed in Table 5.7.

0.0075 M was selected as minimum concentration, 0.06 M was selected as middle concentration and 0.2 M was selected as maximum concentration for this study. Related table was showed below.

Table 5.8.1. Intraday and interday values for retention time of BAC

Concentration (M)	Retention Times ± % RSD	Retention Times ± % RSD
Intraday	*C_{12} Peak*	*C_{14} Peak*
0.0075 M	9.773 ± 0.550	10.093 ± 0.511
0.06 M	9.532 ± 0.760	9.857 ± 0.787
0.2 M	9.630 ± 0.338	9.980 ± 0.309
Interday	*C_{12} Peak*	*C_{14} Peak*
0.0075 M	9.538 ± 4.950	10.162 ± 1.007
0.06 M	9.400 ± 4.270	9.977 ± 1.183
0.2 M	9.530 ± 4.085	8.946 ± 2.091

The relative standard deviation of the intraday and interday test results were between 0.550 – 0.338 % and 4.085 – 4.950 % for C_{12} peak, 0.309 – 0.787 % and 1.107 – 2.091 % for C_{14} peak. The method was found precise.

5.9. Linearity and Range Studies

At the final of optimization studies optimum conditions for the method developed as following: Electrolyte solution was 75 mM pH 6.0 phosphate buffer with 30% ACN, separation potential was 10 kV, and the temperature was 18°C. Samples were introduced by hydrodynamic injection mode by using 50.0 mbar pressure during 3 s. Detection was performed at 214.2 nm due to the maximum absorbance value was obtained at this wavelenght for BAC.

These studies were applied for linearity and the range of concentration (LOD, LOQ).

Table 5.9.1. Calibration curve points

Standard Solution	**Concentration (µg/ml)**	
	C_{12} Peak	**C_{14} Peak**
Standard 1	1.962	0.984
Standard 2	3.270	1.640
Standard 3	6.540	3.280
Standard 4	9.810	4.920
Standard 5	19.620	9.840
Standard 6	32.700	16.400
Standard 7	58.860	29.520
Standard 8	65.400	32.800
Standard 9	98.100	49.200
Standard 10	163.500	82.000

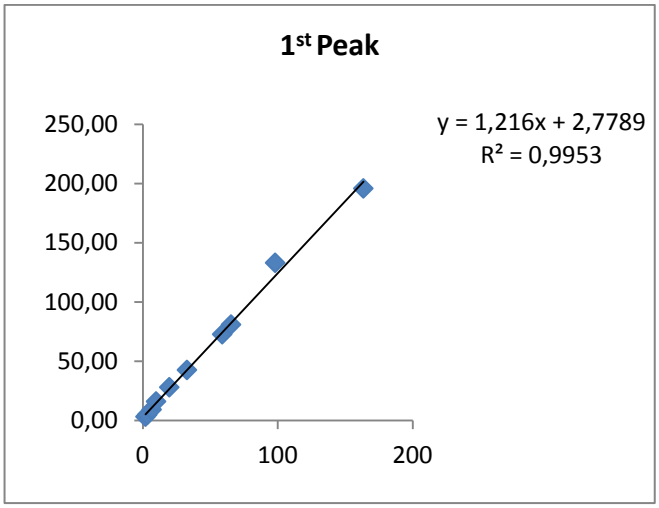

Figure 5.9.1. The calibration curve of BAC for C_{12} Peak

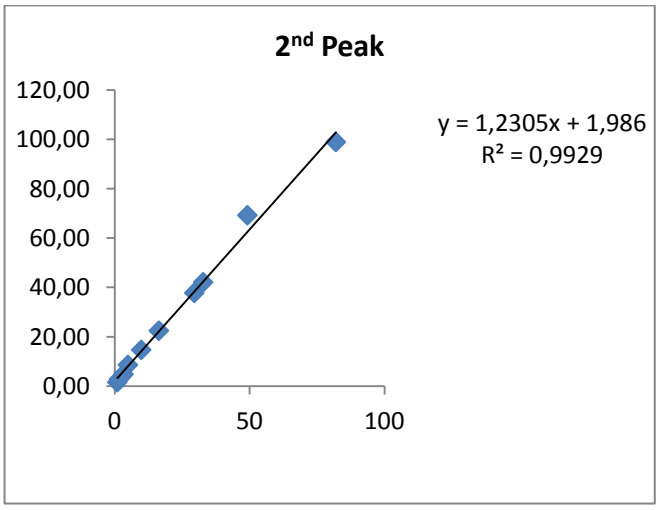

Figure 5.9.2. The calibration curve of BAC for C_{14} Peak

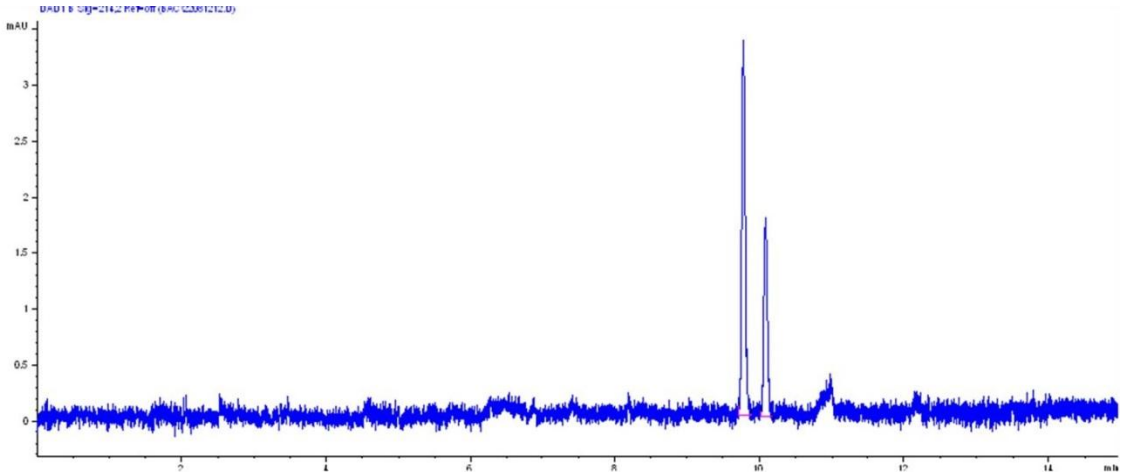

Figure 5.9.3. The typical electropherogram of BAC at optimized conditions

The optimized conditions were found to be 75 mM phosphate buffer with 30% ACN at pH 6.0, and the sample injection of up to 3 s at 50 mbar, and an applied voltage 10 kV. The UV detector is operated at 200 nm and electrophoresis runs were performed at temperature 18 °C. The measured concentration of BAC was 2.5 mg/ml.

BAC – C_{12} peak (1st peak) and BAC – C_{14} peak (2nd peak) were nicely separated from each other and the peaks of them were determined specifically from the main compound.

Table 5.9.2. Calculations of Limit of Detection and Limit of Quantification

Blank signals	Blank signals Value	Standard Deviation of Blank Signals	LOD ug/mL	LOQ ug/mL
b_1	0.13			
b_2	0.11			
b_3	0.12	0.1267	1st Peak: 0.313	1st Peak: 1.042
b_4	0.11		2nd Peak: 0.309	2nd Peak: 1.029
b_5	0.13			
b_6	0.16			

Table 5.9.3. Calibration curve parameters

Calculation Parameter	**C_{12} Peak**	**C_{14} Peak**
Dynamic Range (µg/mL)	1.962 – 165.500	0.984 – 82.000
Slope	1.216	1.231
Intercept	2.779	1.986
LOD (µg/mL)	0.313	0.309
LOQ (µg/mL)	1.042	1.029
R^2	0.9953	0.9929

5.10. Robustness and Ruggedness of the Method

For robustness and ruggedness of the method, parameters optimized were changed delibaretely as declared in ICH. Parameters changed are shown in Table 5.10.1.

Table 5.10.1. Robustness and Ruggedness Parameters

Parameter	**The Lower Value**	**The Higher Value**
ACN %	28	32
pH	5.8	6.2
Wavelength	212.2	216.2

At first, the ratio of ACN was changed. The method was performed using electrolyte with ratio of 32% ACN, 28% ACN. And then the pH values were changed from 5.8 to 6.2.

Finally, the wavelength was changed values as 212.2 and 216.2 nm also. All values and results are as follows.

Table 5.10.2. Robustness and Ruggedness Tests Results

Conditions	Concentrations (M)	C_{12} Peak		C_{14} Peak	
		Recovery (%)	**RSD (%)**	**Recovery (%)**	**RSD (%)**
% 28 ACN	*0.0075*	135.20	1.16	139.00	1.60
	0.06	89.15	1.83	88.52	1.324
	0.2	107.12	3.08	106.29	3.41
% 32 ACN	*0.0075*	78.50	3.50	74.64	2.78
	0.06	105.70	3.49	105.54	3.82
	0.2	98.30	3.88	98.50	3.39
pH 5.8	*0.0075*	84.83	2.55	71.16	3.42
	0.06	97.07	0.12	99.43	1.50
	0.2	95.05	5.81	97.62	6.24
pH 6.2	*0.0075*	82.16	2.28	82.71	1.37
	0.06	99.28	5.80	102.05	5.82
	0.2	84.25	3.35	84.73	3.55
Wavelength 212.2 nm	0.0075	78.63	3.05	75.92	4.02
	0.06	103.12	1.93	103.52	2.09
	0.2	95.13	5.33	94.86	5.41
Wavelength 216.2 nm	*0.0075*	74.03	4.40	74.33	6.40
	0.06	103.27	1.97	103.66	1.96
	0.2	96.53	4.95	95.91	4.89

Robustness and ruggedness of the test results were found precise. Maximum RSD% values were 5.80 for C_{12} peak and 6.40 for C_{14} peak.

5.11. Sample Analysis

Sample solutions were prepared as described in Section 4.2.

Firstly, wetting solutions were studied. To determine the amount of adsorbed BAC on dry wipe, wetting solution containing 0.1 % BAC was treated on dry wipe samples in ratio of 2.80 (w/w) (used value to moisture the dry wet wipes in commercial products) and the wipes were allowed to stand at room temperature for a week. Then wet wipes were wringed and obtained solution was diluted by adding methanol. The dry wipe adsorption capacity solution was used directly for measurement.

The studies were investigated in wet wipes without any extraction and only they were diluted. Then wet wipes were studied and this calculation was used in this study. Wet wipes were wringed and then sample solutions were diluted by methanol.

The typical electropherogram of solutions are presented below.

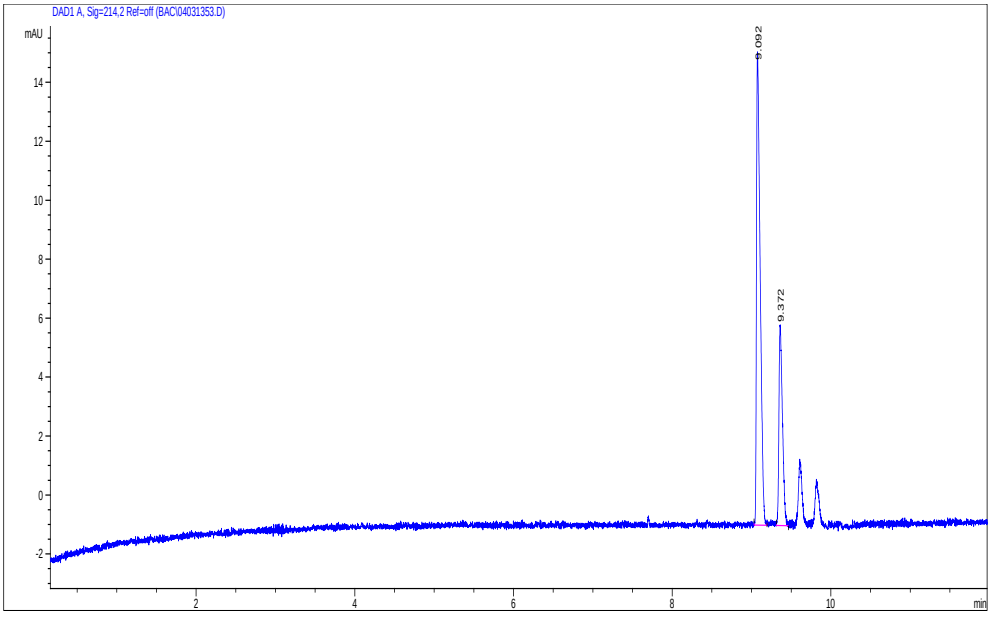

Figure 5.11.1. The typical electropherogram of wetting solution

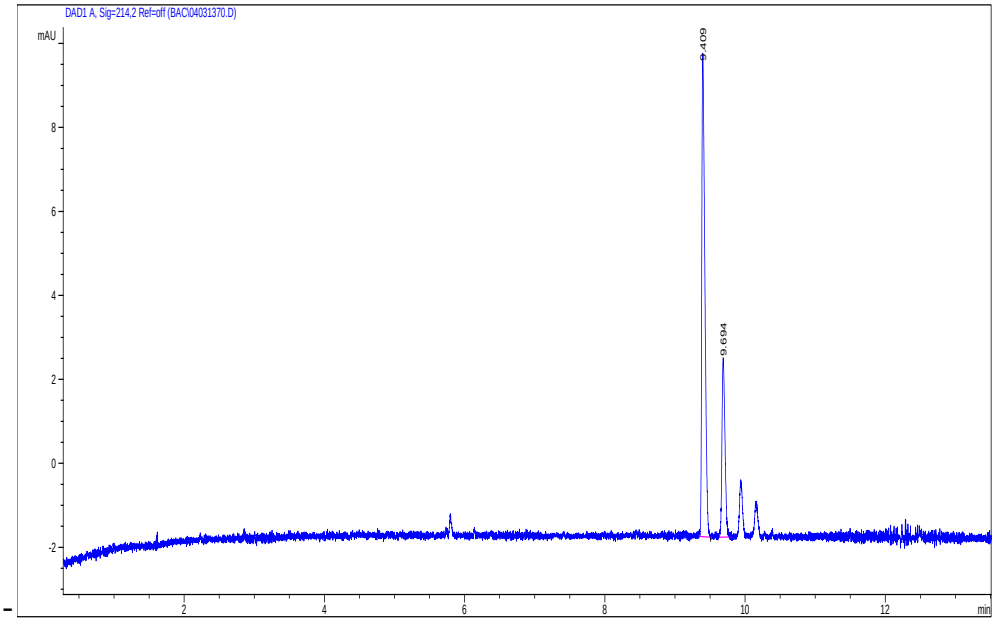

Figure 5.11.2. The typical electropherogram of dry wipe adsorption capacity solution

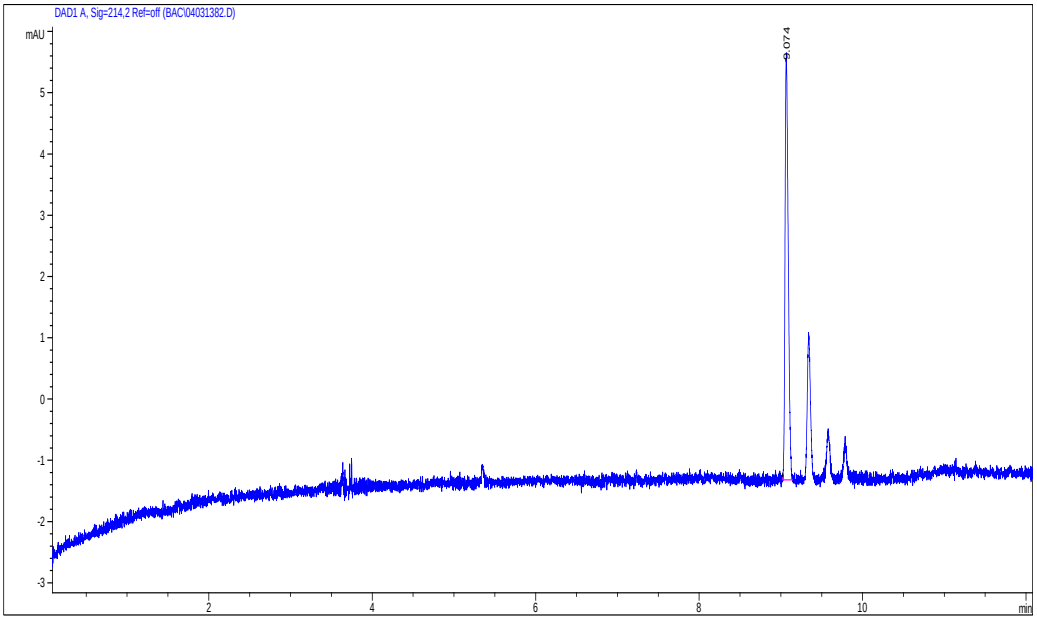

Figure 5.11.3. The typical electropherogram of sample solution

Also, a commercial surface cleaning solution containing BAC was analyzed by this CE method and the results were obtained properly. Related electrophoregram and table are below.

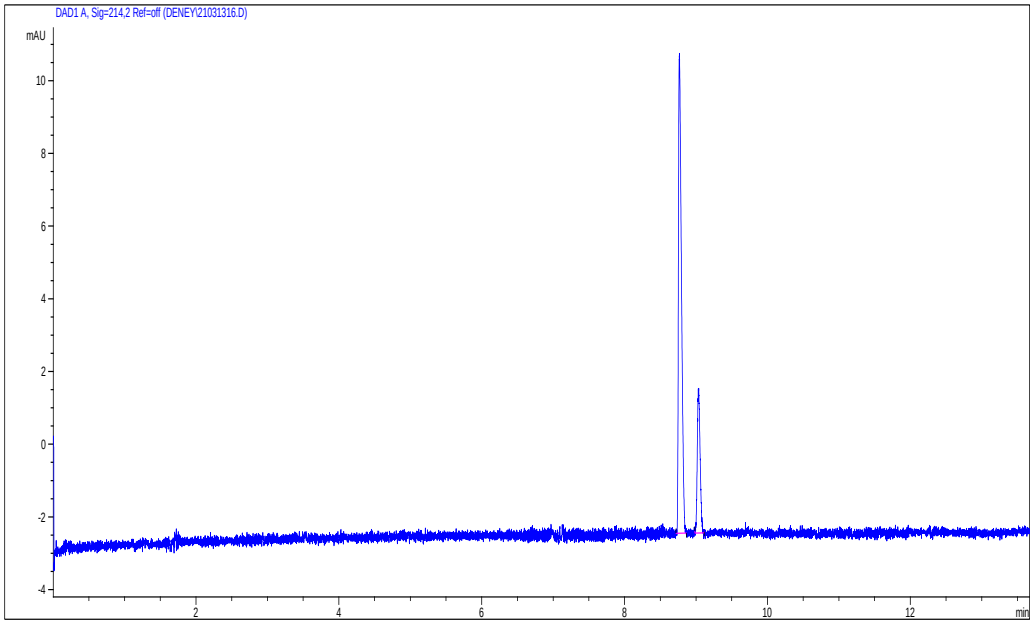

Figure 5.11.4. The typical electropherogram of a biocidal product containing BAC

Table 5.11.1. Wet wipe recovery table

Sample	Study	Claimed Amount of BAC		Found amount of BAC		Error %	
		C_{12} Peak (mg/mL)	C_{14} Peak (mg/mL)	C_{12} Peak (mg/mL)	C_{14} Peak (mg/mL)	C_{12} Peak (mg/mL)	C_{14} Peak (mg/mL)
Wet wipe 1	1	0.280	0.115	0.266	0.084	0.050 %	26.957 %
	2	0.280	0.115	0.224	0.053	20.000 %	53.913 %
	3	0.280	0.115	0.316	0.096	12.857 %	16.522 %
Wet wipe 2	1	0.280	0.115	0.213	0.073	23.926 %	36.522 %
	2	0.280	0.115	0.272	0.107	2.857 %	6.957 %
	3	0.280	0.115	0.316	0.086	12.857 %	25.217 %
Wet wipe 3	1	0.280	0.115	0.224	0.076	20.000 %	33.913 %
	2	0.280	0.115	0.286	0.063	2.143 %	45.217 %
	3	0.280	0.115	0.345	0.102	23.214 %	11.304 %

The wet wipes containing BAC were analysed by the method. The most successful result was second study for wet wipe 2.

A biocidal product containing BAC was analysed by the same method. The method was applied for this product. The results were presented below.

Table 5.11.2. A biocidal product containing BAC

Sample	**Claimed Amount of BAC**	**Found amount of BAC**	**Error %**
Biocidal product	5 g/100 mL	5.4 g/ 100 mL	8.00 %

The results of test for the biocidal product were found precise.

6. CONCLUSIONS

In this study, the capillary electrophoresis method that used for the quantitative determination of wet wipes that include BAC has been developed and validated. Identification and quantitation of homolog of BAC in wet wipes that include BAC substance is applied by using this method. Satisfactory validation data was achieved for sensitivity, linearity, precision, recovery, robustness and ruggedness.

The BAC standard was found to be linear within the concentration ranges. The correlation coefficient was 0.9953 for C_{12}, 0.9929 for C_{14}. The concentration ranges of BAC are accordance with literatures.

Two peaks that belongs to BAC is determined by using this method. The retention time of C_{12} peak is about 9.6 minute and the retention time of C_{14} peak is about 9.8 minute. The retention times were short.

In wet wipes claimed amounts of BAC had to be, 0.280 mg/ml for C_{12} and 0.115 mg/ml for C_{14}. Also; the found amounts of BAC were 0.274 mg/ml for C_{12} and 0.082 mg/ml for C_{14}.

A biocidal product containing BAC was analysed by the same method. In this product claimed amount of BAC had to be 5 g/100 ml and the found amount of BAC was 5.4 g/ml.

According to the other methods, the most important advantage of this method is to only done by diluting without having to any pre-treatment priorly such as extraction in wet wipes containing 0.1% of the concentration of BAC.

The wet wipe solution which is including 0.1% BAC has also essentiol oil and preservative, beside BAC. Having clean and clear electropherogram result, in this kind of complex substance, is very important quality of this method.

7. REFERENCES

[1] Xue Y, Hieda Y, Saito Y, Nomura T, Fujihara J, Takayama K, Kimura K, Takeshita H. Distribution and disposition of benzalkonium chloride following various routes of administration in rats. Toxicology Letters, 148: 113-123, 2004.

[2] Pauloina T, Dutota M, Warneta J, Rata P. In vitro modulation of preservative toxicity: High molecular weight hyaluronan decreases apoptosis and oxidative stress induced by benzalkonium chloride. Eur. J. of Pharmaceutical Sciences, 34: 263-273, 2008.

[3] Pernak J, Mirska I, Kmiecik R. Antimicrobial activities of new analogues of benzalkonium chloride. Eur. J. Med. Chem. , 34: 765-771, 1999.

[4] European Pharmacopeia. (7th ed.) 0372: 1461-1463, 2009.

[5] Smith M.J, Flowers T.H, Cowling M.J, Duncan H.J. Method for the measurement of the diffusion coefficient of benzalkonium chloride, Water Research, 36: 1423–1428, 2002.

[6] Turku I, Sainio T. Modeling of adsorptive removal of benzalkonium chloride from water with a polymeric adsorbent, Separation and Purification Technology, 69: 185–194, 2009.

[7] Troy D.B, Beringer P. Remington: The Science And Practice Of Pharmacy. (21st ed.) Lippincott Williams & Wilkins, pp 1628, 2006.

[8] Iwasawa A, Ayaki M, Niwano Y. Cell viability score (CVS) as a good indicator of critical concentration of benzalkonium chloride for toxicity in cultured ocular surface cell lines, Regulatory Toxicology and Pharmacology, 66: 177–183, 2013.

[9] Deutschle T, Porkert U, Reiter R, Keck T, Riechelmann H. In vitro genotoxicity and cytotoxicity of benzalkonium chloride. Toxicology in Vitro, 20: 1472-1477, 2006.

[10] The United States Pharmacopeia. (29th ed.), 2006.

[11] Gaber M, Shawish H.M.A, Khedr A.M, Abed-Almonem K.I. Determination of benzalkonium chloride preservative pharmaceutical formulation of eye and ear drops using new potentiometric sensors. Mater. Sci. Eng. C 32: 2299-2305, 2012.

[12] Kovács-Hadady K, Fábián I. The determination of benzalkonium chloride in eye-drops by difference spectrophometry. J. Pharm. Biomed. Anal. , 16: 733-740, 1998.

[13] Bernal J.L,Nozal M.J, Martin M.T, Diez-Masa J.C, Cifuentes A. Quantitation of active ingredients and excipients in nasal sprays by high-performance liquid chromatography, capillary electrophoresis and UV spectroscopy, J. Chromatogr. A, 823: 23–431, 1998.

[14] Miyauchi T, Mori M, Ito K. Quantitative determination of benzalkonium chloride in treated wood by solid-phase extraction followed by liquid chromatography with ultraviolet detection. J. Chromatogr. A, 1095: 74-80, 2005.

[15] Cybulski Z. R. Determination of benzalkonium chloride by gas chromatography, J. of Pharmaceutical Sciences, 73: 1700, 1984.

[16] Dudkiewicz-Wilczyńska J, Tautt J, Roman I. Application of the HPLC method for benzalkonium chloride determination in aerosol preparations. J. Pharm. Biomed. Anal. , 34: 909-920, 2004.

[17] Kümmerer K, Eitel A, Braun U, Hubner P, Daschner P, Mascart G, Milandri M, Reinthaler F, Verhoef J. Analysis of benzalkonium chloride in the effluent from European hospitals by solid-phase extraction and high-performance liquid chromatography with post-column ion-pairing and fluorescence detection. J. Chromatogr. A, 774: 281-286, 1997.

[18] Hou Y-H, Wu C-Y, Ding W-H. Development and validation of capillary zone electrophoresis method for the determination of benzalkonium chlorides in ophthalmic solutions. J. Chromatogr. A, 976: 207-213, 2002.

[19] Jimidar M, Beyns I, Rome R, Peeters R, Musch G. Determination of benzalkonium chloride in drug formulations by capillary electrophoresis. Biomed. Chromatogr. , 12: 128-130, 1998.

[20] Para B. V, Núńez O, Moyano E, Galceran M.T. Analysis of benzalkonium chloride by capillary electrophoresis-tandem mass spectrometry. Electrophoresis, 27: 2225-2232, 2006.

[21] Altria K.D, Elgey J, Howells J.S. Validated capillary electrophoretic method for the quantitative analysis of histamine acid phosphate and/ or benzalkonium chloride. J. Chromatogr. B, 686: 111-117, 1996.

[22] Xu Y. Tutorial: Capillary Electrophoresis. The chemical educator, 96: 1430-4171, 2006.

[23] Skoog A, West M, Holler J, Crouch R. Fundemantals of Analytical Chemistry. (8th ed.) Brooks Cole, 2003.

[24] Watson D. Pharmaceutical analysis-a textbook for pharmacy students and pharmaceutical chemists, (2nd ed.) Elsevier Science Health Science Division, 2005.

[25] Altria K.D. Overview of capillary electrophoresis and capillary electrochromatography. J. Chromatogr. A, 856: 443-463, 1999.

[26] http://en.wikipedia.org/wiki/Van_Deemter_equation

[27] Tagliaro F, Turrina S, Smith F.P. Capillary electrophoresis: principles and applications in illicit drug analysis. Forensic Sci. Int. , 77: 211-229, 1996.

[28] Li S.F.Y. Capillary Electrophoresis, Principles, Practices and Applications, Elsevier Science Publishers. 52: pp 14, 1992.

[29] Validation of analytical procedures: text and methodology Q2(R1), ICH Harmonised Tripartite Guideline, 2005.

[30] Vriesa P.D, Willemsa E.W, Heiligersa J.P.C, Villalónb C.M, Saxena P.R. The antimigraine agent alniditan selectively constricts porcine carotid arteriovenous anastomoses via 5-$HT_{1B/1D}$ receptors. Eur. J. of Pharmacology, 351: 193-201, 1998.

BIOGRAPHY

Güneş Yıldırım was born in Istanbul on March 26, 1987.

She graduated from Ilhami Ahmed Ornekal Primary School in 2001 and 50. Yıl Tahran High School in 2005.

Since her childhood, she’ s always had an interest about science and technology, by the way this interest caused her to study on chemistry academically.

She graduated from chemistry department of Kocaeli University in 2009 (bachelor' s degree).

In 2011, she applied Yeditepe University for higher education to study on pharmaceutical chemistry at institute of medical sciences.

It has been three years since she started to work at Munir Sahin Medicine Industry as a Research & Development Specialist.

She has been living in Istanbul as the only child of her family.