

SEPARATION OF *N*-NITROSAMINES BY MICELLAR ELECTROKINETIC CHROMATOGRAPHY

(Pemisahan *N*-Nitrosamina Menggunakan Kromatografi Elektrokinetik Misel)

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Abstract

A simple and rapid micellar electrokinetic chromatography (MEKC) method was developed for separation of three selected *N*-Nitrosamines namely *N*-nitrosodipropylamine (NDPA), *N*-nitrosodibutylamine (NDBA) and *N*-nitrosodiphenylamine (NDPhA). The effects of composition of the buffer and its pH, concentration of surfactants on the separation and migration times of nitrosamines were investigated. The instrumental variables affecting sensitivity and resolution such as power supply and injection mode were carefully optimized. The best separation was achieved using 40 mM sodium dodecyl sulfate (SDS) as a surfactant in 10 mM phosphate buffer (pH 8.0) at a temperature of 25 °C, applied voltage of 29 kV, wavelength of 230 nm and electrokinetic injection of 9 s at 5 kV within 10 min analysis time. Excellent linearity was obtained in the concentration range of 2 to 100 µg/mL with coefficients of determination, $r^2 \geq 0.979$. This method showed good reproducibility with relative standard deviation (RSDs) value ranging from 2.46% to 6.61%. The limits of detection (LOD) and limits of quantification (LOQ) ranged from 0.16 to 0.43 µg/mL and 0.54 to 1.44 µg/mL respectively.

Keywords: Nitrosamines, surfactants, capillary electrophoresis, micellar electrokinetic chromatography

Abstrak

Satu kaedah kromatografi elektrokinetik misel yang mudah dan cepat telah dibangunkan bagi pemisahan tiga sebatian *N*-Nitrosamina yang dipilih iaitu *N*-nitrosodipropilamina (NDPA), *N*-nitrosodibutilamina (NDBA) dan *N*-nitrosodifenilamina (NDPhA). Kesan komposisi penimbal dan pH, kepekatan surfaktan terhadap pemisahan dan masa peralihan nitrosamina telah dikaji. Pembolehubah alat yang mempengaruhi kepekaan dan resolusi seperti bekalan kuasa dan mod suntikan telah dioptimumkan dengan berhati-hati. Pemisahan yang terbaik dicapai menggunakan 40 mM SDS sebagai surfaktan di dalam 10 mM penimbal fosfat (pH 8.0) pada suhu 25 °C, voltan gunakan 29 kV, panjang gelombang 230 nm dan suntikan elektrokinetik 9 saat pada 5 kV dengan masa analisis selama 10 minit. Kelinearan yang baik telah diperolehi dalam julat kepekatan 2 - 100 µg/mL dengan koefisien penentuan, $r^2 \geq 0.979$. Kaedah ini menunjukkan kebolehulangan yang baik dengan nilai sisihan piawai relatif dalam julat 2.46% - 6.61%. Had pengesanan dan had penentuan adalah 0.16 - 0.43 µg/mL dan 0.54 - 1.44 µg/mL, masing-masingnya.

Kata kunci: Nitrosamina, surfaktan, elektroforesis kapilari, kromatografi elektrokinetik misel

Introduction

Nitrosamines are a family compounds discovered 100 years ago and received worldwide attention. These compounds were discovered carcinogen in experimental animals. Nitrosamines have been tested positive as mutagenic (chemicals that can change DNA) and carcinogens which target organs such as liver, kidney, lungs, skin and eyes. Nitrosamines are compounds that consist of NNO group and commonly existing in the environment and food. They have been found in many food products [1-2], tobacco smoke [3], rubber [4], water from chlorinated swimming pools [5], wastewater, treated wastewater, groundwater and drinking water [6-9]. Nitrosamines were produced by reaction of amines or their derivatives with nitrosating agents such as nitrous acid, nitrites, or nitrogen oxides. These compounds are relatively stable and difficult to destroy once formed. Nitrosamines have been

detected at ppb levels in a wide variety of matrices. The maximum allowable concentrations levels for nitrosamines in water are generally set at low ng/L. The State of California from California Department of Public Health had issued an action level of 10 ng/L for certain nitrosamines in response to the discovery of *N*-nitrosodimethylamine (NDMA) in some of its drinking water supplies. Therefore, sensitive analytical methods with the lowest possible detection limits become a demand for their determination at trace level amounts.

Various analytical methods have been studied for determination of these compounds. The most common analytical tools used are gas chromatography (GC) [10-12] and liquid chromatography (LC) [13,14] with highly selective and sensitive detector such as mass spectrometry (MS). However, the choice of such detector for the analysis of nitrosamines is often highly costly and not available in most laboratories. In addition, LC system requires relatively large amounts of samples and eluents containing organic solvents and a long time to treat a separation column.

This has encouraged the development a simpler and less expensive methods for nitrosamines determination. Recently, capillary electrophoresis (CE) emerged as a popular separation technique in many applications such as analysis of proteins [15,16], pharmaceuticals [17,18] and environmental pollutants. CE is relatively new when compared to the more seasoned techniques, HPLC and GC. Each technique comes with its own particular strengths and limitations. GC-MS based techniques have been commonly used for regulatory analysis of this compound. But CE has some very attractive features which make it both competitive and a good alternative. In particular, CE is generally applicable to a broad range of analytes (neutral, charged, organic, inorganic) and offers excellent resolving power.

There are a several modes in CE that can be developed for a wide variety of analytes. As nitrosamines are hydrophilic, neutral and polar compounds, a specialized CE technique is required for their separation. CE methods have been developed previously for determination other group of nitrosamines such as tobacco specific nitrosamines using capillary zone electrophoresis (CZE) mode [19-20]. Micellar electrokinetic chromatography (MEKC) is a mode of CE that enables the separation of electrically neutral analytes by electrophoresis in a manner which is analogous to the principles of chromatography. The aim of this work was to develop a methodology for the separation, identification and quantification of selected nitrosamines using CE. *N*-nitrosodipropylamine (NDPA), *N*-nitrosodibutylamine (NDBA) and *N*-nitrosodiphenylamine (NDPhA) were selected as model compounds (Figure 1). The developed methodology was applied to the determination of these analytes in an aqueous sample.

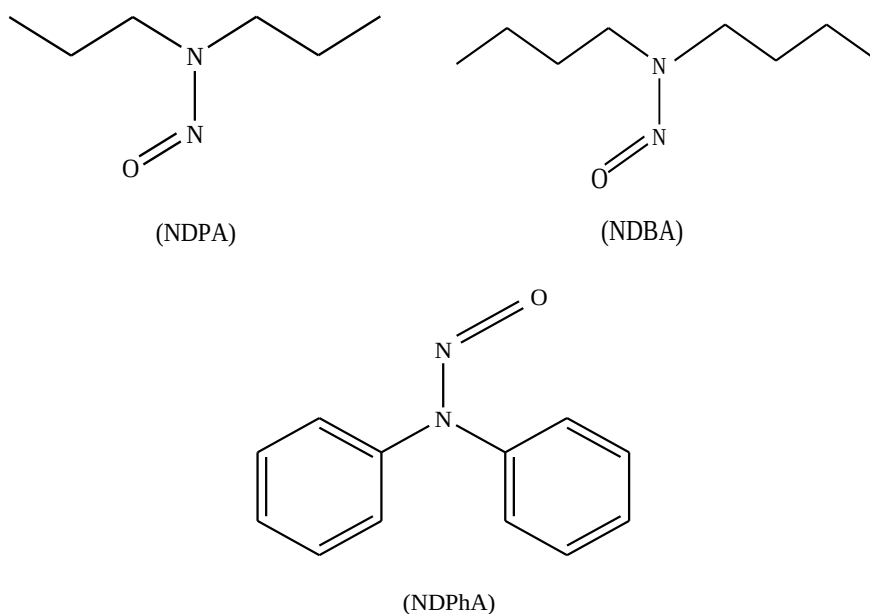


Figure 1. Structures of the NDPA, NDBA and NDPhA used in the development of the CE method.

Materials and Methods

Reagents

Disodium hydrogen phosphate 12-hydrate, sodium dodecyl sulfate (SDS) and sodium deoxycholate (SDC) were purchased from Sigma-Aldrich (St. Louis, MO). Sodium cholate (SC) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) whereas sodium hydroxide was purchased from Riedel-de Haen (Seelze, Germany). NDPA, NDBA and NDPhA were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and solvents were common brands of analytical-reagent grade. The water used for dilutions or as buffer preparation was produced from a Water Purification System from Millipore (Molsheim, France).

Instrumental

All CE separations were conducted on an Agilent Technologies CE (Waldbronn, Germany) equipped with a photodiode array detection system. The MEKC was performed using a 64.5 cm × 50 µm i.d. uncoated fused-silica capillary with an effective length of 56 cm obtained from Polymicro Technologies (Phoenix, AZ). Samples were injected by using electrokinetic injection (EKI) and detection wavelength used was 230 nm. The CE system was controlled by a PC workstation using Chemstation software.

Electrophoresis conditions

Prior to the first use, a new capillary was conditioned by passing 1 N NaOH solution for 30 min followed by washing with deionized water for 30 min and finally equilibrating with an appropriate running buffer for 30 min. Between runs, the capillary was washed with 0.1 N NaOH, water and run buffer for 10 min each.

Preparation of running electrolytes

Running electrolytes were prepared by dissolving surfactants at an adequate concentration in phosphate buffer where phosphoric acid was used to adjust the desired pH.

Standard solution preparation

A 2000 µg/mL stock standard solution of each analyte was prepared in methanol and stored in the refrigerator in the dark. A working standard solution was made daily by diluting stock with water. The sample to be injected was at a typical concentration of 200 µg/mL. All the separation solutions were filtered through 0.20 µm nylon syringe filter obtained from Whatman (Clifton, NJ, USA).

Results and Discussion

Optimization of electrophoretic conditions

The running electrolyte conditions, such as type and concentration of the surfactant also buffer composition significantly affect the electrophoretic separation. Therefore, before selecting the conditions for optimization, a number of preliminary trials were conducted with different type of surfactants and buffer composition at various pH to check the migration time, area, shape and resolution. The electrophoretic parameters were initially evaluated using a 50 mM SC surfactant and 50 mM borate buffer (pH 7.0). The use of this buffer did not give adequate separations (Figure 2). The compounds migrated very close to the electroosmotic flow (EOF) and the peaks overlapped. Then, in another attempt the use of buffer consisting of 50 mM SDS and 50 mM phosphate buffer (pH 7.0) as a running electrolyte was evaluated. The background electrolyte (BGE) of SDS and phosphate buffer in this ratio was found to be more appropriate since all the desired peaks were obtained. Hence, this BGE was chosen for further optimization. The peaks for each analyte were identified by analysis of individual analytes at typical concentrations of 200 µg/mL.

Effect of pH, Buffer and Surfactant Concentration

The initial CE separations were carried out at pH 7.0. At this condition, separations of the three nitrosamines peak were achieved within 12 min. Various buffer pHs in the range of 6.0-8.0 was studied by adding phosphoric acid into the solution. A small change of pH can have a dramatic effect on migration time and peak area of analytes. At pH 6.0, long migration times were achieved at 24 min. The change in the buffer pH especially in the lower pH region caused a significant change in the velocity of EOF. Therefore, the electroosmotic mobility was reduced in the acid range and this led to long migration time [21]. Meanwhile, at pH 8.0, high peak areas (>288 mAu) were obtained

as compared to those at pH 7.0 (< 123 mAu). As nitrosamines studied were in ionized form and has the same charge as the micelle, they were incorporated into the micelle to a smaller degree than their neutral forms. Therefore, more analyte distribution occurs at the anionic SDS at pH 8.0. Hence, pH 8.0 was selected as the best pH for the further optimization. Figure 3 shows a comparison of the peak areas of the analytes at different pHs.

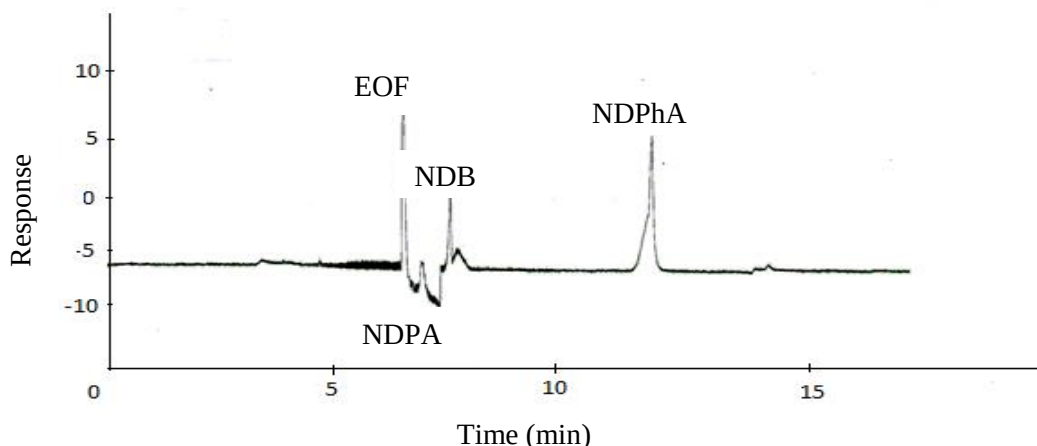


Figure 2. Electropherogram of a nitrosamine standard mixture at 200 µg/mL, before the optimization steps. Separation conditions: 50 mM sodium cholate in 50 mM borate buffer (pH 7); capillary 64.5 cm × 50 µm I.D (effective length, 56 cm); applied voltage, 20 kV; temperature, 25 °C; Injection electrokinetic, 5 kV/5 sec.

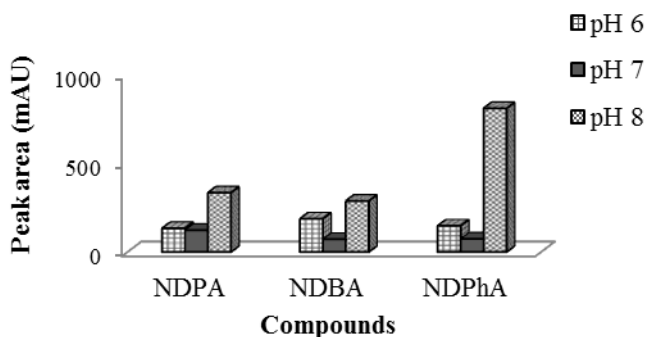


Figure 3. Study effect of pH on the MEKC of nitrosamines at 200 µg/mL. Separation conditions: 50 mM SDS in 10 mM phosphate buffer; capillary 64.5 cm × 50 µm I.D (effective length, 56 cm); applied voltage, 20 kV; temperature, 25 °C; Injection electrokinetic, 5 kV/5 sec.

To analyze the effect of SDS concentration, it was varied from 30-60 mM. Optimization SDS played a key role in achieving efficient separation in MEKC because it worked as a pseudostationary phase where neutral nitrosamines species partition themselves between the micelles and buffer solution. The optimized concentration of SDS was 40 mM as it gave a good peak area (Figure 5). Further increase the SDS concentration to 60 mM tend to peak become broad due to long migration times. From the Figure 4, it shows that migration time was further increased when concentration SDS was increased. The increased in SDS concentration caused a significant interaction between the analytes with a micelles. Viscosity of solution will also increase, lead to an increase in their migration times.

Different phosphate buffer concentrations in the range of 5-15 mM were investigated. The ionic strength of buffer varies as the concentration was changed. Increased buffer concentration in BGE caused an ionic strength that modulated the EOF and electrophoretic mobility of the analyte. Thus, the migration times of nitrosamines became longer as concentration of phosphate buffer was increased. Further increase in buffer concentration led to peak broadening that is probably due to Joule Heating effect [22]. Therefore, 10 mM was chosen as the optimum buffer concentration as it gave faster migration times than that for 15 mM and better resolution as compared to that for 5 mM.

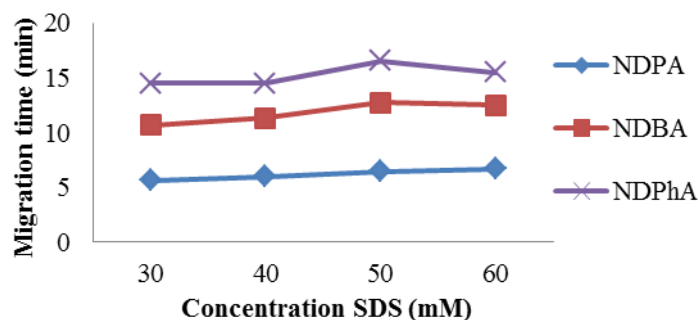


Figure 4. Effects of SDS concentration on the migration time of nitrosamines at 200 µg/mL. Separation conditions: 10 mM phosphate buffer (pH 8.0); capillary 64.5 cm × 50 µm I.D (effective length, 56 cm); applied voltage, 20 kV; temperature, 25 °C; Injection electrokinetic, 5 kV/5 sec.

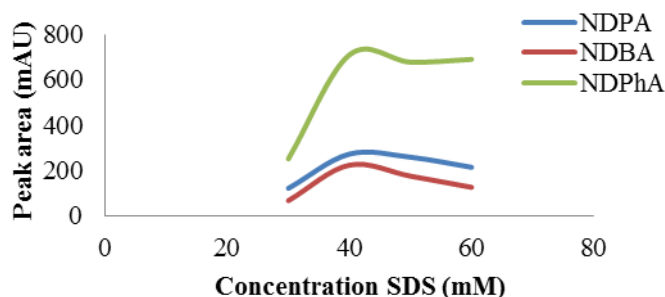


Figure 5. Effects of SDS concentration on the area of nitrosamines at 200 µg/mL. Separation conditions: 10 mM phosphate buffer (pH 8.0); capillary 64.5 cm × 50 µm I.D (effective length, 56 cm); applied voltage, 20 kV; temperature, 25 °C; Injection electrokinetic, 5 kV/5 sec.

Effect Injection Time, Applied Voltage and Temperature

After optimizing the buffer conditions, instrumental variables namely applied voltage, temperature, injection mode and injection were also studied and optimized. Effect of different applied voltages (20, 23, 26 and 29 kV), injection times (3, 5, 7 and 9 sec) and temperatures (15, 20 and 25 °C) on the separation of nitrosamines was investigated. Varying the applied voltage and temperature gave a significantly changing migration times. Analysis time was decreased with increase in applied voltage. In this study, 29 kV was the maximum voltage that can be reached by the system; higher applied voltage of > 29 kV will cause instrumental current increase. Meanwhile, the optimum temperature obtained was 25 °C which is the maximum temperature that can be reached when 29 kV voltages was applied.

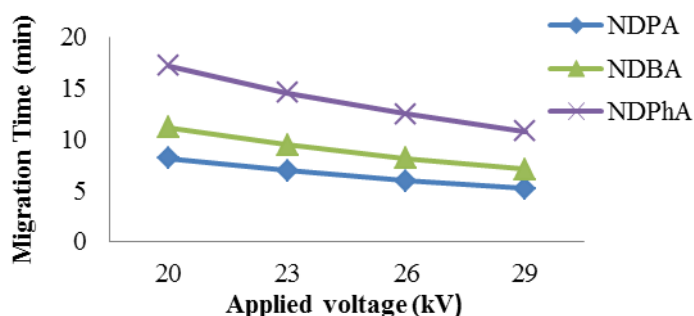


Figure 6. Study effect of applied voltage on the migration of nitrosamines at 200 $\mu\text{g/mL}$. Separation conditions: 40 mM SDS, 10 mM phosphate buffer (pH 8.0); capillary 64.5 cm $\times$ 50 μm I.D (effective length, 56 cm); temperature, 25 $^{\circ}\text{C}$; Injection electrokinetic, 5 kV/5 sec.

In this study, electrokinetic injection was used. No separation was observed when hydrodynamic injection was used. Electrokinetic injections at 5 kV and 9 sec was found to be the best conditions achieved as all peak were resolved and high peak areas obtained.

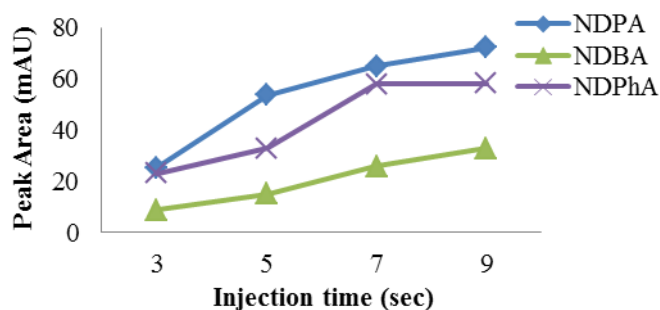


Figure 7. Study effect of injection time the area of nitrosamines at 200 $\mu\text{g/mL}$. Separation conditions: 40 mM SDS, 10 mM phosphate buffer (pH 8.0); capillary 64.5 cm $\times$ 50 μm I.D (effective length, 56 cm); applied voltage, 29 kV; temperature, 25 $^{\circ}\text{C}$; Injection electrokinetic, 5 kV.

An electropherogram after the optimization of all these variables is showed in Figure 8 and the optimum conditions are summarized in Table 1. Electrophoretic method was characterized by constructing a calibration graph without any preconcentration of sample in the concentration range of 2-100 $\mu\text{g/mL}$. The calibration curves with peak area versus concentration were plotted and the curves obtained were linear for three analytes in the mentioned concentration ranges with coefficient of determination, $r^2 \geq 0.979$. The limit of detection (LOD) was calculated using 3-times the standard deviation of the intercept divided by the slope, whereas the limit of quantification (LOQ) was calculated by using 10 times the standard deviation of the intercept divided by the slope. LOD were found to be in the range 0.16 to 0.43 $\mu\text{g/mL}$ and LOQ in the range 0.54 to 1.44 $\mu\text{g/mL}$. This method was found to be reproducible with relative standard deviation (RSD) for intra day precision less than 6.61%. The analytical performances of MEKC method for the determination of nitrosamines are summarized in Table 2.

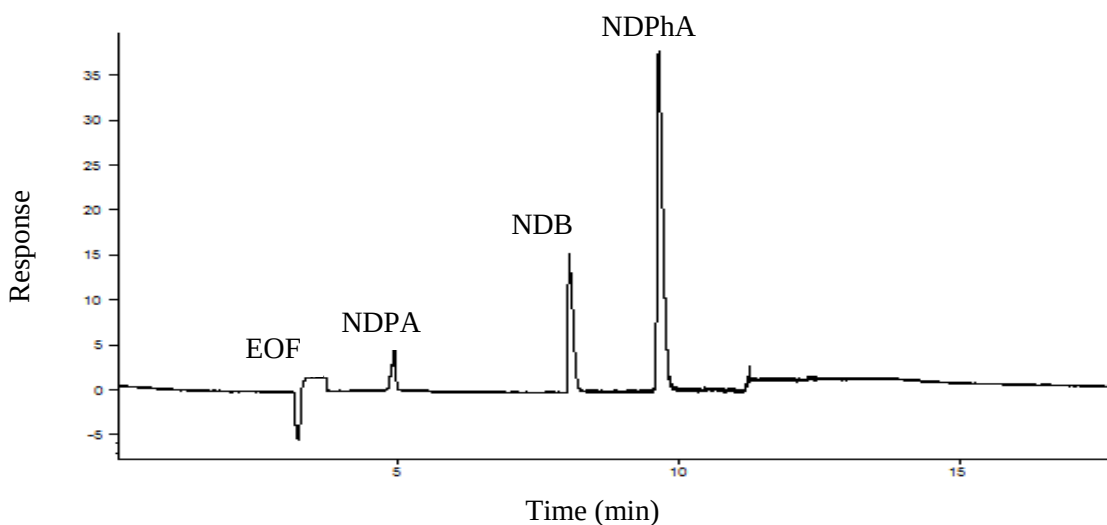


Figure 8. Electropherogram of a nitrosamine standard mixture at 200 µg/mL, after the optimization steps
Separation conditions: 40 mM SDS in 10 mM phosphate buffer (pH 8); capillary 64.5 cm × 50 µm I.D (effective length, 56 cm); applied voltage 29 kV; temperature, 25 °C; Injection electrokinetic, 5 kV/9 sec.

Table 1. Optimization parameters and optimized conditions for the development of MEKC method

Parameters	Studied	Optimized
Surfactants	SC SDS SDC	SDS
Concentration SDS	30-60 mM	40 mM
Buffer	Borate Phosphate	Phosphate
Concentration phosphate	5-15 mM	10 mM
pH	6-8	8
Injection mode	Hydrodynamic Electrokinetic	Electrokinetic
Injection time	3-9 sec	9 sec
Applied voltage	20-29 kV	29 kV
Temperature	15-25 °C	25 °C

Table 2. Analytical Performance of MEKC method

Analyte	Equation	r^2	Linear range ($\mu\text{g/mL}$)	LOD ($\mu\text{g/mL}$)	LOQ ($\mu\text{g/mL}$)	% RSD peak area (n=3)
NDPA	$y = 0.2343x + 0.6127$	0.998	2-100	0.43	1.44	2.46
NDBA	$y = 0.7955x - 1.3367$	0.999	2-100	0.36	1.19	5.30
NDPhA	$y = 2.5679x - 15.006$	0.979	2-100	0.16	0.54	6.61

y = peak area, x = concentration ($\mu\text{g/mL}$)

The developed method was then applied for determination of these three selected compounds in aqueous sample. Dispersive micro-solid phase extraction (D- μ -SPE) technique using multiwall carbon nanotubes (MWCNTs) has been carried out for the extraction of these three selected nitrosamines. The standards solution was spiked in distilled water and undergoes D- μ -SPE. Triplicate analysis was performed on 5, 10 and 15 $\mu\text{g/mL}$ for the precision study. Percent of recovery was calculated by comparing the area before and after the extraction of the aqueous sample. The recoveries obtained for representative nitrosamines was summarize in the Table 3. High recovery obtained only for NDPA and NDBA (between 80.3 % and 97.2 %). However, low recovery obtained for NDPhA may due to the strong π - π interaction of analyte with the MWCNTs used in extraction. The developed method was simple as the separation by CE equipment just required a low cost of capillary and less volume consumption for BGE. In addition, this method also led to significant environmental benefits due to solvent-free consumption in BGE. In the present study, it was a first time separation of these three selected nitrosamines by using MEKC method. Therefore, the analysis time within 10 min was considered rapid and good for separation of neutral, polar compound nitrosamines by MEKC.

Table 3. Analysis of aqueous samples by the proposed method (concentration in $\mu\text{g/mL}$)

Analyte	Amount added ($\mu\text{g/mL}$)	Amount founded ($\mu\text{g/mL}$)	Recovery (%)
NDPA	5	4.86	97.2
	10	8.88	88.8
	15	13.26	88.4
NDBA	5	4.40	88.0
	10	8.22	82.2
	15	12.05	80.3
NDPhA	5	2.63	52.6
	10	4.81	48.1
	15	7.81	52.1

Conclusion

In the present study, MEKC method was carried out to determine the best optimized conditions for separation of three selected nitrosamines. It can be concluded that this method is simple and rapid using commercial CE equipment. The results obtained were acceptable and suitable for separation and determination of nitrosamines in aqueous sample at $\mu\text{g/mL}$ concentration.

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