



Research Article

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Liquid Chromatographic and Validation Study in Separation and Determination of Benzidines and Phenols in the Main Discharge Point of Wastewater

Bahaa Malik Altahir^{1*}, Muntadhar Salih Sultan², Amer Saleh Mahdi² and Hadi Hassan Jasim²

¹Biology Department, College of Science, University of Baghdad, Baghdad, IRQ

²Chemistry Department, College of Science, University of Mustansiriyah, Baghdad, IRQ

ABSTRACT

Benzidines and phenols are the most priority pollutants. Separation and quantitative estimation of priority pollutant benzidines composed of various benzidines BZ, including substituted 3, 3'-dichlorobenzidine DCB and 3, 3'-dimethylbenzidine DMB, and priority pollutant phenols (9 compounds, i.e., phenol, 2- and 4-nitrophenol, 2,4-dimethylphenol, 2-, 2,4-di-, 2,4,6-tri-, Penta-chlorophenol, and 4-chloro-3-methylphenol) was performed using high performance liquid chromatography-ultra violet techniques. Both groups were separated using a C-18 column with a UV detector at a wavelength of 280 nm, and the flow of the mobile phase was isocratic. The mobile phase consisted of 75:25 methanol: water. The column temperature was 50°C, and the flow rate was 1.8 ml/min for the Benzidine's separation. The mobile phase consisted of a 50:50 acetonitrile: phosphate buffer. The optimum pH was 7.1, the flow rate was 0.7 ml/min and the optimum column temperature was 45°C for the phenols separation. The separation parameters were calculated, including the chromatographic parameters such as the capacity factor (k), the number of theoretical plates (N), the selectivity factor (α), and the resolution factors (Rs). This method was applied to real samples. The water samples that were analyzed were obtained from a petroleum refinery wastewater treatment unit. The results ranged between undetectable levels and 246.9 $\mu\text{g/L}$ of the selected benzidines. The results were ranged between undetectable levels and 1865.61 $\mu\text{g/L}$ of the selected phenols.

Keywords: Chromatographic study; Petroleum refinery wastewater; Benzidines; Priority pollutant

Phenols; HPLC

INTRODUCTION

Benzidine-based azo dyes are widely used in the dye manufacturing, textile dyeing, color paper printing, and leather industries [1]. Benzidine and its derivatives have been used to manufacture dyes for many years [2] It is classified as a known human carcinogen by the Environmental Protection Agency (EPA), the National Toxicology Program (NTP), and the International Agency for Cancer Research (IARC) [3]. Many of these dyes find their way into the environment via wastewater facilities. These dyes also exhibit a high resistance to microbial degradation in wastewater treatment systems [4]. Phenol and its derivatives are common pollutants found in effluents from industrial operations dealing, for example, with coal conversion, pulp and paper manufacturing, wood preservation, metal casting,

and production of pesticides, and they are rated as priority pollutants by the US EPA (code U188)[5]. The presence of phenolic compounds in soils is due to different sources, including industrial activities related to the chemical, textile, pharmaceutical, polymers, pulp and paper, woods, plasticizers, pesticide, and metallurgic industries or by the release of industrial effluents and domestic sewage [6]. Phenols are easily accumulated in soils and can contaminate the environment. Given increasing concerns on environmental quality, several countries have established strict limits on acceptable environmental levels of phenols [7].

The content of benzidinic compounds in aqueous samples can be determined by various analytical instrumental methods, such as a gas chromatography (GC)/mass spectrometric assay [8], rapid colourimetry [9], fluorescence spectrographic method [10], novel resonance light scattering (RLS)[11], ion selective electrodes [12], direct injection and Ultrahigh-pressure liquid chromatography (UPLC) with fluorescence detection[13], and high-performance liquid chromatography with various detectors such as UV detector [14], mass spectroscopy detector[15] electrochemical detector [16]

The content of phenolic compounds in aqueous samples can be determined by various analytical instrumental methods such as gas chromatography (GC) /mass spectrometric assay [7] spectrophotometric determination [17,18], electro analytical and potentiometric analysis [19], high performance liquid chromatographic with various stationary phases as well as, hyper-cross linked polystyrene resin with multiple flow modes, including isocratic and gradient flow (have been employed with various detectors such as diode-array[20], mass spectrometry [21] ultraviolet detection [22].

Benzidines were determined in different environmental matrices such as water [15] and river samples[12]. Phenolic compounds were determined in various samples, such as environmental [22], food [19], Agricultural [21] seawater, soil, sediments [7,17,18], and wastewater samples[20].

HPLC is the preferred technique for priority pollutant aromatic compounds separation, because it is simple, robust, reliable, accurate and highly selective [23].

In addition, pretreatment and extraction step is necessary prior to HPLC to remove interfering components. The proposed method is simple and rapid and practical for the identification and simultaneous determination of several compounds in a short period of time. Studies have been carried out to determine the amount of benzidines and phenols in industrial waste water and environmental water samples, but little previous study has investigated these compounds in petroleum refinery waste water as well as previous full chromatographic study. The Dora oil, petroleum refinery station is one of the most important stations in Iraq and possesses a wastewater treatment unit. The oil, petroleum refinery waste water is a source of aromatic compounds because these compounds are the major components of crude oil[24]. The aromatic compounds may affect living organisms in water. Benzidines and PPP are hazardous pollutants and aromatic compounds that can be found in petroleum waste water. One of the aims of this study is to investigate the concentrations of benzidines and PPP in water based on treatment stages.

METHODOLOGY

Materials

The standard solution of phenols [i.e., 604 Phenols Calibration Mix] and the Standard solution of Benzidines [Benzidine, 605 benzidines calibration mix (BZ, DCB), 8270 benzidines mix (BZ, DCB, DMB)] were supplied by Restek Chromatography Product and Solutions.

All solvents were HPLC grade and supplied by (sigma-Aldrich (St. Louis, MO, USA), Himedia Laboratories (Mumbai, India) and J.T Baker (Netherland). All of the other chemicals were of analytical grade with a purity >98.0%. These chemicals were obtained from Sigma Chemical Company Inc., Aldrich Chemical Corporation (Milwaukee, WI) or BDH Inc. The CHROMABOND® SPE cartridges {HR-P (polystyrene-divinylbenzene) (PS-DVS), octadecyl silica (ODS), Cyclohexyl silica (CHS)} were supplied by the MACHEREY-NAGEL Company.

Instruments

A Shimadzu HPLC (LC-20AD), DGU-20As degasser, LC 20A four pumps P, N 7725i Sample injector, Shimadzu SPD-20A prominence UV-VIS detector, and Column Oven CTO 20AD were employed. The column (EC Nucleodur C₁₈RP) Stainless steel made with a length of 250 mm and an inner diameter of 4.6 mm (Macherey Nagel). The column protection system (EC guard column, holder system) had an inner diameter of 2 mm (Machinery Nagel Co.). A special syringe made for HPLC (M. SYRINGFE, 122F-LC) was used for the injection of the 20 µl samples in the mobile phase and onto the column. The extraction system was designed to manually extract different samples at the same time.

Temperature Effect

The effect of temperature was optimized. The Organic solvent was 75:25 methanol: Water with a flow rate of 1ml/min and different temperatures (40, 45, 50, 60°C) were investigated. The results indicated that a decrease in the retention factor occurred as the temperature increased. In general, an increase in the column temperature reduced the retention factor [26]. However, the *k* values of BZ and DMB did not change substantially, but that of DCB did exhibit a larger change. However, a small decrease in *k* was observed with BZ from 45-50°C and 50-60°C that which may be due to the high viscosity of the eluent (i.e., 75% methanol). The optimum temperature was 50°C because the *k* values were not as close to each other, resulting in molecules that are more separated with a shorter analysis time Figure 2

Flow Rate

The flow rate was optimized. The optimum conditions, including 75% organic solvent (methanol) and an oven temperature of 50°C were held constant. Different flow rates (i.e., 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.2, 2.4, and 2.6 ml/min) were used to separate the mixture. The results are shown in Figure 3. A decrease in the *k* values of BZ, DCB and DMB from 1-1.4 ml/min was observed. In contrast, an increase in the *k* values from 1.4-2.6 was observed for all of the benzidines. The optimum flow rate was 1.8. This flow rate was selected because the *k* values were not as close to each other and the molecules were more separated with a shorter analysis time. Moreover, a flow rate greater than 1.8 causes deterioration of the separation column. A small effect of the flow rate on the separation was observed. A decrease in the *k* values was observed as the analysis time decreased, which may be due to an increase in the partitioning equilibrium between the mobile phase and the stationary phase.

Detection

The wavelength (λ) changes (i.e., 230, 250, 280, 310, and 340 nm) were studied under the optimum separation conditions. The peak area was used as an indicator to choose the best wave length. A wavelength of 280 nm was optimal, as shown in Figure 4

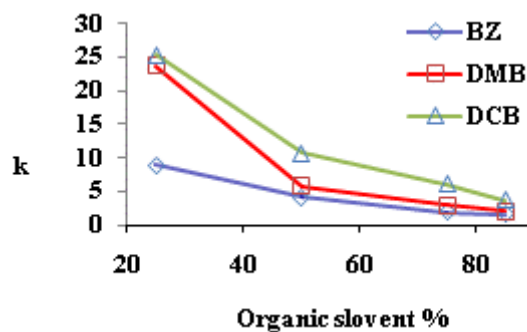


Figure 1: Organic solvent percentage and *k* values of BZ, DCB, and DMB (Flow rate of 1 ml/min at 50°C)

Figure 2: Temperature effect and *k* values of BZ, DCB, and DMB (Flow rate of 1 ml/min with 75: 25 organic solvent)

Figure 3: Flow rate and k value of BZ, DCB, and DMB(Temperature of 50°C 75: 25% with organic solvent)

Figure 4: Peak areas and wave lengths of BZ, DCB, DMB under the optimum conditions with 10000 µg/L

Optimization of the Purification and Separation of Priority Pollutant Phenols (PPPs) by HPLC

Selection of Mobile Phase: Organic Solvent modifier Percentage in Aqueous Phase

The organic solvent percentage was optimized using mixtures of acetonitrile and water with different organic solvent percentages (15, 25, 35, 50 and 60%) as the mobile phase. The pH of the aqueous portion was the same as that of DIW 7. In addition a flow rate of 0.7 ml/min, and a column temperature of 45°C were held constant during the separation of the PPPs by HPLC. The k values were calculated. The results are shown in Figure 5 Most of the studied phenols were highly sensitive to increases in the organic solvent (i.e., acetonitrile (ACN)) percentage. The optimum organic solvent percentage was 50 % because the k values were not as close to each other in addition, the convergence of k values at a lower value resulted in better separation of the molecules, and the analysis time was shorter.

pH Effect

The pH of the mobile phase, which contains 50% organic solvent (acetonitrile) was optimized using a, column temperature of 45°C and a flow rate of 0.7 ml/min. Different pH values [i.e.3.7, 5.2 (acetate buffer), 6.6, 7.1, and 7.6 (phosphate buffer)] were used to separate the mixture. The results are shown in Figure 6 Change in the k values was observed for most of the PPPs. The optimum pH was 7.1 because the convergence of the k values was to a lower value. In addition the molecules were more separated. The pKa values of the phenol are 9.95 while (7.14-7.23) for nitrophenols and (8.48-9.32) for chlorophenols [27]. The pKa values indicate that approximately 50% of the chlorophenols are in an ionic form [unprotonated] and 50% are protonated. The other phenols such as the nitro phenols are unprotonated. The difference in the [unprotonated] / [protonated] forms yields the difference in the partitioning equilibrium between the mobile phase and the stationary phase, which aids in the full separation of these compounds. The retention factor of the partially ionized compound can be predicted in reversed-phase liquid chromatography by eqn. (1)

$$k = k_o + k_i \frac{k_a + k_i \left(\frac{k_a}{H^+} \right)}{1 + (k_a / (H^+))} \quad \dots \quad (1)$$

Figure 7: Flow rate and k value of PPP (Temperature of 45°C, organic solvent 50:50)

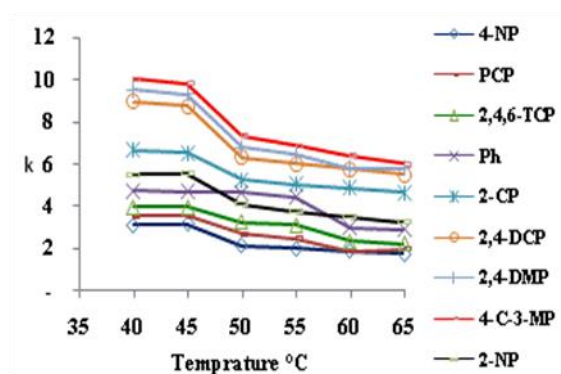


Figure 8: Temperature and k values of PPP (Flow rate of 0.7 ml/min, organic solvent 50:50)

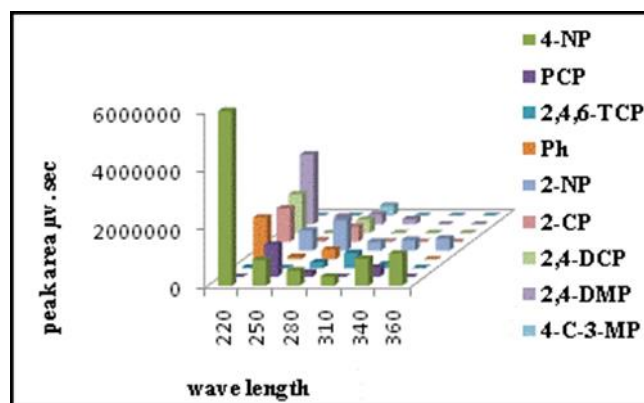


Figure 9: Wavelengths and peak areas of PPPs under optimum separation conditions

HPLC Separation Parameters

The retention time (t_R) and void time (t_M) were used to calculate the retention factor (k) of the eluted benzidines and phenols using the following equation $k = (t_R - t_M) / t_M$ [28, 29] were obtained from the chromatograms in Figures 10 and 11. The number of theoretical Plates (N) was computed using the HPLC parameter equation. ($N = 16(t_R/w)^2$) The selectivity factor (α) was computed using the following equation: $\alpha = k_2/k_1$, In addition the resolution factor was calculated using the following equation $R_s = 2(t_2 - t_1) / (w_1 + w_2)$, and the Tailing factor was calculated using the following equation

2,4,6-TCP	250-5000	0.9966	14.34	3913.0	35.56	118.5
Ph	250-5000	0.9995	32.06	2180	145.21	484.04
2-NP	250-5000	0.9993	90.3	3365.1	155.69	518.99
2-CP	250-5000	0.9961	40.1	365.2	196.82	656.08
2,4-DCP	250-5000	0.9979	42.43	1496.8	226.22	754.09
2,4-DMP	250-5000	0.9971	25.98	-109.78	102.31	341.03
4-C-3-MP	250-5000	0.9995	20.096	1251.3	127.70	425.69

Analytical Method Validation (Precision, accuracy, ruggedness and robustness)

The precision (in terms of the relative standard deviation) was calculated via the repeatability. The accuracy (in terms of the relative error percent) was calculated via the recovery percentages. Triplicate analysis was performed for any single benzidines or phenols at three concentrations chosen from the high, low and middle levels within the Beer's law range on the standard curve. The precision and accuracy results for benzidines and phenols are shown in Tables 3-6.

The ruggedness of benzidines (Intraday) was determined by triplicate injections at a single concentration (i.e., 800 µg/L) on 3 days using the average of 3x3 injections. The results for the ruggedness were 2.93, 3.65, and 3.36 for BZ, DMB and DCB, respectively. The robustness was calculated by triplicate injections of a single 800 µg/L standard solution after incubation in an oven at 50°C for 30 min. The mean, standard deviation, and RSDs were calculated.

The ruggedness of phenols (Intraday) was determined by triplicate injections of a single concentration (5000) µg/L within 3 days using average of 3x3 injections. The ruggedness was 1.95, 3.36, 1.61 for BZ, DCB and DMB, respectively. The robustness was calculated by triplicate injections of a single 5000 µg/L standard solution after incubation in an oven at 50°C for 30 min.

Table 1: Analytical method validations (Precision, accuracy, ruggedness and robustness)

Benzidine	RSD%	Er%	Ruggedness	Robustness
BZ	1.81	2.64	2.93	3.26
DMB	1.04	1.30	3.65	2.99
DCB	1.78	1.02	3.36	4.35

Table 2: Analytical method validations (Precision, accuracy, ruggedness and robustness)

Phenol	RSD%	Er%	Ruggedness	Robustness
4-NP	5.31	-3.53	3.37	3.31
PCP	3.36	5.52	2.82	3.02
2,4,6-TCP	3.42	4.53	3.19	4.69
Ph	1.47	-2.14	3.04	3.08
2-NP	1.76	1.07	2.82	1.55
2-CP	2.15	1.49	3.67	2.80
2,4-DCP	3.25	3.47	3.41	4.89
2,4-DMP	2.91	4.80	3.16	2.63
4-C-3-MP	3.02	2.70	4.00	2.42

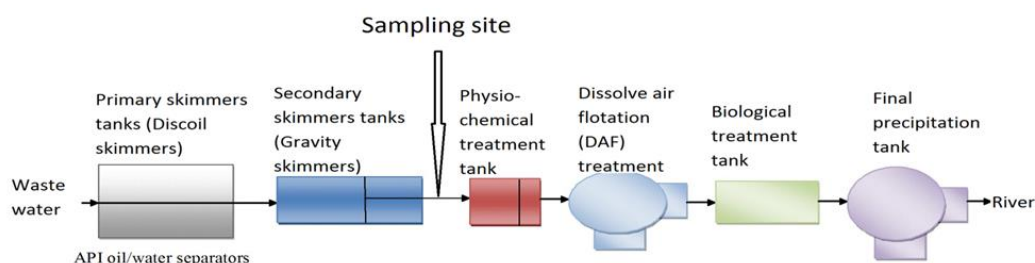
Petroleum Refinery, Industrial Waste Water Treatment Stages

The Dora petroleum refinery station is located south of Baghdad in Iraq. The end stream waste water from this station is discharged into the Tigris River at the end of the treatment process. This waste water is one source of aromatic compounds that can enter the river and affect the health of living organisms in the river. Therefore the amount of priority pollutant compound in the waste water treatment unit should be evaluated. Benzidines and phenols are priority pollutant compounds that can be found in petroleum waste water because aromatic compounds are the main part of petroleum oil. The station contains a waste water treatment subunit that includes many treatment subunits, including skimmer subunits, a physiochemical subunit, DAF subunit, and biological subunit.

The waste water streams are collected in the main tanks to gather all of the waste water coming from the various stages of the refinery stations (heavy and light oil). The stream goes down to the first treatment subunit (skimmers and discoil skimmers) in this stage, and the subunit separates the oil phase from the water phase.

In the second stage, the stream reaches to the physiochemical subunit tank. Many chemicals, such as poly electrolyte and alum are added to this tank, to aid in aggregation of the semi-soluble materials to separate these materials using special skimmers in the next stage (i.e., dissolved air floatation (DAF tank)). In the third stage, the stream goes down to the next subunit (i.e., the biological treatment tank). In this treatment, bacteria are used to biodegrade the organic and inorganic molecules. Phosphoric acid and urea are added to this tank, and a flotation ventilator aids in the re-oxygenation of the bacteria. The last tank, (i.e., the final precipitation tank) collects the final waste water prior to entering the river.

Phenols and benzidines were determined and followed monthly for one year at a specific site. This site was located after the skimmer subunit. The sampling was conducted using a special pump between the skimmer and physiochemical subunits. The samples were pre-filtered, extracted and eluted by solid-phase extraction under extraction conditions mention above. The eluted samples were purified, separated, detected and measured by HPLC –UV under optimum conditions. The eluted samples were injected in triplicate, and the mean and standard deviation, relative standard deviation were calculated. The samples were diluted to 1:2 and 1:5 to decrease the viscosity and the concentration of the samples were evaluated within the range of detection.



Scheme 1: Stages of waste-water treatment in the petroleum refinery station with sampling sites A, B, and C

Determination and Monthly Variation Study of PPB

Most benzidines were observed in most of the samples. The results are shown in Figure 12. In general, the amounts of the benzidines selected in this study were lower in the spring and autumn than during the other seasons. The highest results were 155.44 $\mu\text{g/L}$, 264.99 $\mu\text{g/L}$ and 131.12 in Feb, for BZ, DMB and DCB, respectively. In March, March, and Jan. BZ, DMB and DCB, respectively, were Not detected

Determination and Monthly Variation Study of PPPs

Most PPPs were observed in most of the samples. The results are shown in Figure 13. The amounts of the PPPs selected in this study were typically lower in the summer than during the other seasons. The highest results were 1034.37 $\mu\text{g/L}$ in Oct., 1018.61 in Oct. and 1136.33 in Jun. for 2,4,6-TCP, 2,4-DCP and Ph, respectively. During several months, these compounds were not detected.

The variation in the levels of these compounds may be caused by the operation conditions, volatilization, dilution, and evaporation because the tanks are exposed to air and sunlight.

