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Method Development for Analysis of Phthalates by HPLC

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For the Degree of Masters of Science,

With a Major in Analytical Chemistry

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Abstract:

The purpose of this study is to develop an analytical method for determining the concentration of dialkyl phthalates as part of a larger study of phthalate degrading bacteria. Specifically, bacteria isolated from polyaromatic hydrocarbon contaminated riverine sediment are being determined for their proficiency in degrading dimethyl phthalate, diethyl phthalate, and dibutyl phthalate." HPLC instrument was used for analysis of these phthalate derivatives. A reversed phase isocratic and gradient elution and UV detection method was used for the individual and simultaneous determination of these phthalate derivatives. HPLC was carried out using a C18 column and spectrophotometric detection at 230nm. An isocratic elution was performed using methanol–water (75/25 %) as a mobile phase. Standard calibration curves were linear for all three analytes.

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Introduction:

Phthalates are the most widely used plasticizers in plastic industry.¹ Phthalates are dialkyl or alkyl aryl esters of 1, 2-benzene dicarboxylic acid. These plasticizers are not chemically bound to the plastics. Therefore, they can leach from the materials and come contact with surroundings, resulting in ubiquitous environmental contamination. Since phthalate exposure is considered to potentially have adverse effects on human health, analytical methodology for measuring their migration into food and beverages has been extensively reported.¹ Also, diethyl phthalate (DEP) and dibutyl phthalate (DBP) are used in personal care products, lacquers, varnished and coatings. Ingestion, inhalation and dermal contact are considered important routes of exposure to phthalates in the general population.

HPLC stands for “High Performance Liquid Chromatography.” HPLC is sometimes referred to as High-Pressure Liquid Chromatography. HPLC is a chromatographic technique that can separate a mixture of compounds. It is a powerful tool in analysis which is used in biochemistry and analytical chemistry to identify, quantify, and purify the individual components from the mixture.

There are two modes of operation in the HPLC system:

Normal phase: In normal-phase chromatography, columns packed with polar stationary phases combined with nonpolar or moderately-polar mobile phases are used to separate the components of mixtures

Reverse phase: In reverse phase chromatography, a non-polar substance that achieves sufficient packing is used as a stationary phase. For example, an octadecyl carbon chain bonded with silica (C18 column) or alumina. Polar solvents such as water, methanol, acetonitrile, etc. are used as the mobile phase and are mixed with water.

There are two elution methods for separating the mixture of components:

¹ Bablu, S. Sumathi; Biodegradation of Phthalate Esters by *Variovorax* Sp; *APCBEE Proc. 1*; 2012, 16-21.

Isocratic elution: In this method, the composition of mobile phase remains constant throughout the run. The isocratic mode is most commonly used for assay, dissolution, and routine analysis where one can separate at least 2 or 3 compounds in a single run.

Gradient elution: In this method, the composition of mobile phase varies. Generally gradient modes are useful when you want to analyze a mixture of compounds having differences in polarity that cannot be separated by isocratic elution method.

Parameters:

Column: The success or failure of an analysis depends on the choice of column.² The column's stationary phase separates the sample components using various physical and chemical parameters. The column was selected is Phenomenex column (Figure 2).



Figure 1. Column Phenomenex Synergi 4 μ fusion - RP 80A

Mobile phase ratio: In reversed-phase HPLC (RP HPLC) one of the eluent components is water, which does not interact with the hydrophobic adsorbent surface. It has minimal competition with the analyte for the adsorption sites. The composition of the mobile phase impacts mostly retention time and the shape of the peak.

Temperature: Retention in HPLC is temperature-dependent. If temperature during analyses varies, it is difficult to assign “peaks” to specific compounds in the chromatogram and the peak areas/heights may vary. Thus, a common practice is to use a thermostated column under isocratic condition.

Flow rate: The volume of mobile phase passing through the column in unit time. A faster flow, however, gives the peak less time to diffuse with good separation. Decrease in flow rate results in higher retention time, broader peaks and poor separation.

² United States Environmental protection Agency; Method 8000D: Determinative Chromatographic separation; Revision 4, July 2014.

Materials:

Dimethyl phthalate (DMP), diethyl phthalate (DEP) and dibutyl phthalate (DBP) were purchased from VWR. Methanol, 99.9 % HPLC grade, was purchased from Fisher Scientific. Millipore filter paper (pore size 0.45µm, Whatman filter paper) to purify all the solutions. Ultrasonicator (VMR International, 75 HT) to degas all the solutions that are used for analysis.

Methods:

A stock solution (1×10^{-2} M) was prepared for all three compounds (DMP, DEP, and DBP). Concentrations of 3 mM, 5 mM, and 7 mM standard samples for all three compounds were prepared from the stock solutions. Some amount of these solutions was filtered through a Millipore filter (0.45µm, filter syringes) and transferred into an HPLC vial. All the samples were placed in the HPLC instrument (Agilent 1260 HPLC, ChemStation LC software (C.01.05 [35], 2010-2013)) and run according to the analytical conditions listed below.

Analytical conditions:

Column: stainless steel (150 mm x 4.6 mm) from Phenomenex Synergi 4 µ Fusion – RP 80A (Agilent Technologies)

Flow: 1.0 mL/min

Mobile phase ratio: isocratic, methanol: water (75:25)³

Temperature: 30°C

Injection volume: 10 µL

Wavelength: 230 nm with reference to 360 nm

Run time: For DMP: 5 mins with 2 mins of post time; DEP: 7mins with 2 mins of post time; DBP: 10 mins with 2 mins of post time.

³ D. De Orsi, L. Gagliardi; An environmentally friendly reversed-phase liquid chromatography method for phthalates determination in nail cosmetics; *Anal. Chim. Acta*; 555, 2006, 238-241.

Results:

Dimethyl Phthalate:

As DMP is more polar than other two phthalate derivatives, it elutes first with lower retention time.

The retention time was 2.4 mins and the result for the calibration curve are shown in Table 1 and Figure 2.

Concentration (mM)	Area (mAu)
3.0	1.14
5.0	2.04
7.0	2.76

Table 1. Calibration plot for DMP; concentration vs. absorbance.

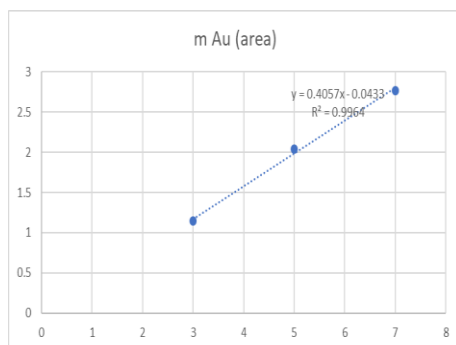


Figure 2. Calibration plot for DMP with concentration on x-axis and area on y-axis.

Diethyl Phthalate:

DEP is less polar than dimethyl phthalate as it contains ethyl group where the number of carbons is increased and it elutes second in series.

The retention time was 2.9 mins which is 0.5 mins longer than DMP.

Concentration (mM)	Area (mAu)
3.0	1.11
5.0	2.36
7.0	3.50

Table 2. Calibration plot for DEP; concentration vs. absorbance.

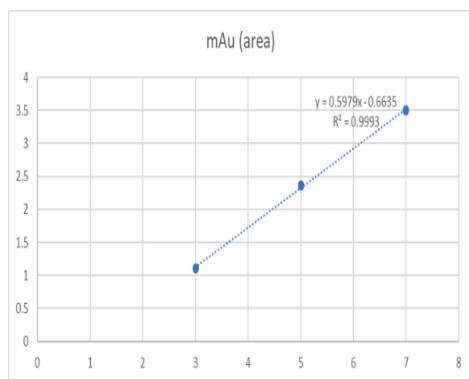


Figure 3. Calibration plot for DEP with concentration on x-axis and area on y-axis.

Dibutyl Phthalate:

DBP is much less polar than DMP and DEP as it contains butyl group and has the longest elution time of the phthalates in the study.

The retention time was 7.2 mins which is approximately 5 mins longer than DMP and DEP.

Concentration (mM)	Area (mAu)
3.0	1.00
5.0	2.06
7.0	3.07

Table 3. Calibration plot for DBP; concentration vs. absorbance.

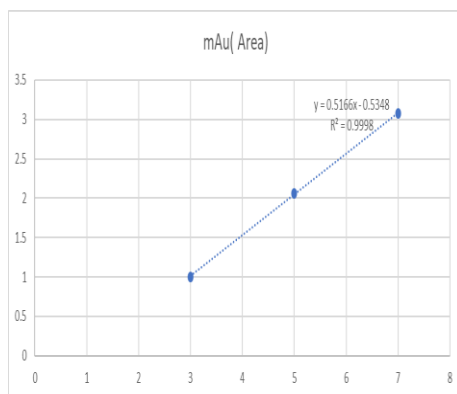


Figure 4. Calibration plot for DBP with concentration on x-axis and area on y-axis.

Discussion:

The main aim of this experiment is to develop a method for analysis of these phthalate derivatives (DMP, DEP, and DBP) by HPLC. HPLC is routinely used for both qualitative and quantitative analysis of environmental, pharmaceutical, industrial, forensic, clinical, and consumer product sample. Because, a fixed volume sample loop provides a more precise and accurate injection, quantitative HPLC is easier than other analysis. As a result, most quantitative HPLC methods use external standards and a normal calibration curve. In this experiment, we used isocratic elution method with mobile phase ratio [methanol: water (75:25)] for analysis, as the sample containing only one component without any mixture.

Initially different amounts of concentrations [methanol: water (80: 20, 90: 10 and 60; 40)] was performed for analysis, but, retention time was longer and broad peaks was appeared. As previously mentioned, success and failure of analysis depends on the choice of the column. I used column (Phenomenex Synergi 2.5 μ fusion – RP 80A) for separation of the components. Due to the short length of the column, the peaks eluted at lower retention time with improper separation. The peaks got well separated with column (Phenomenex Synergi 4 μ fusion - RP 80 A) with good retention time and was used for analysis of standard samples. The detection was performed both 220 nm and 230 nm, however, 230 nm was selected due to higher sensitivity. Flow rates (0.5, 1.0, 1.5 ml/min) for analysis was performed. High flow rate (1.5ml/min) resulted in a reduction in retention time, but also poor separation was observed. The components have little time to interact with stationary phase and eluted by the column quickly. Low flow rate (0.5 ml/min) resulted in peak broadening as the components had more time to stay on stationary phase and high retention time was observed. Flow rate (1.0 ml/min) was selected for analysis, it gave a good separation of peaks with lower retention time. Finally, temperature (25, 30, 35°C) was applied for analysis of the components. Retention time in HPLC is temperature dependent. An excessively high column temperature results in very short retention time but also in a very poor separation. However, for the separation to occur the components need to be able to interact with the stationary phase. If the compound does not interact with the stationary phase, the retention time will decrease. Temperature 35°C was not applied because, it was difficult to assign “peaks” to specific compounds in the chromatogram and the peak areas/heights varied. Temperature 30°C was applied for analysis of the components, as it gave a lower retention time with good separation of peaks.

A calibration curve was plotted for all the samples from obtained results. All three samples show linearity in the calibration curve. Linearity can be evaluated by the visual inspection of a plot of signals obtained in the results. The linear relationship can be evaluated by statistical methods by calculating correlation coefficient, slope of regression line, and y-intercept.⁴ From the above results, correlation coefficient is positive, which was greater than 0.995. If the concentration

⁴ International Conference on Harmonization; Validation of Analytical procedures: Text and Methodology Q2 (R1), revision 4, 2005.

increases, area also increases, and in negative correlation, if there is a decrease in concentration, area also decreases.⁵ Furthermore, validation procedure like limit of detection (LOD,⁶ limit of quantification (LOQ).⁶ can be calculated based on linear regression. Linear regression has an equation form of:

$$Y = a + bX$$

Where a = intercept

b = slope of the line

y = dependent variable

X = explanatory variable

For the limit of detection, the lowest detectable signal is calculated from the intercept and standard error of the regression line.

$$\text{LOD} = 3.3 \sigma / s$$

Where σ = the standard deviation of y-intercept of regression line

s = slope of calibration curve.

For limit of quantification, the lowest detectable signal is calculated from the intercept and standard error of the regression line⁷.

$$\text{LOQ} = 10 \sigma / s$$

Where σ = the standard deviation of y-intercepts of regression lines

s = slope of calibration curve

Conclusion:

As per the chromatogram obtained during the analysis of the phthalate derivatives confirms that dimethyl phthalate with low retention time elutes faster than diethyl and dibutyl phthalates. In future studies, I recommend performing the analysis for these phthalate derivatives by using 5 μ column to examine if there is better resolution within retention time of the chromatogram. If less resolution is required, there will be increase in particle size⁸.

⁵ Critical Care20037:451 <https://doi.org/10.1186/cc2401> © BioMed Central Ltd 2003, Published: 5 November 2003

⁶ N. Jornet-Martínez, C. Antón-Soriano, Estimation of the presence of unmetabolized dialkyl phthalates in untreated human urine by an on-line miniaturized reliable method, *Sci.of the Environ.*;532,2015,230-244.

⁷ Olivieri, A.C.; Practical guidelines for reporting results in single and multi-component analytical calibrations: A tutorial; *Anal. Chim. Acta*; 868, 2015, 10-22.

⁸ <http://pubs.acs.org/doi/pdf/10.1021/jo00408a041>

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