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Method Development for Determination of Di(2-Ethylhexyl)Phthalate and its Metabolite Mono(2-Ethylhexyl)Phthalate by Reversed-Phase Liquid Chromatography

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ABSTRACT

Di(2-ethylhexyl)phthalate (DEHP) is a benzenedicarboxilate ester which is the most widely used plasticizer to soften resins. DEHP is hydrolyzed into what many believe to be its toxic metabolite, mono(2-ethylhexyl)phthalate (MEHP), by lipase enzymes in the gastrointestinal tract. The reversed-phase high-performance liquid chromatographic method to determine the presence of DEHP and MEHP simultaneously was developed. Using several methodological variables including controlled pH of the mobile phase, inclusion of different solvents for the sample, and introduction of an ion-pairing reagent, the best method for quantification of both chemicals was determined to be linear gradient elution. The estimated detection limits were determined to be 3 and 2 ppm for DEHP and MEHP, respectively. The developed method was found to be simple and precise, with a high degree of accuracy for determining DEHP and its metabolite.

INTRODUCTION

According to I. Paris et. al. (2003), Di(2-ethylhexyl)phthalate (DEHP) is one of the most widely used plasticizers for polyvinylchloride (PVC). Lundberg and co-workers (1992) estimated the world production of DEHP at approximately 1 million tons per year. Hill et. al. (2001) explains that DEHP essentially acts as a lubricant between the polymeric chains and causes the flexibility and softness of the plastic at low temperatures. Both Paris et. al. (2003) and Lundberg et. al. (1992) conclude that the DEHP content in PVC can be up to 40% (w/w). As a result of the plasticity and durability provided by DEHP, PVC is used in many applications such as construction material, waterproof clothing, food-wrapping material, industrial tubing, toys, medical devices (catheters, blood storage bags, tubing), cosmetics, and dieletric fluid in condensers, as stated by Lundberg et. al., (1992) Arcadi et. al (1998), and Kato et. al (2003). Despite these often-desired characteristics, concerns about the interactions between the plastic and the contained materials, such as food, blood, and pharmaceuticals are well documented. These interactions include sorption, the uptake of the containing material by the plastic, and leaching, the

release of plastic additives into the containing material, as described by Jenke (2003). Various concentrations have been found in food due to the DEHP leaching from packaging. Levels of 13.4-91.5 mg/kg (dry weight) have been reported in lung tissue of patients that received transfusions. Notably, Lundberg et. al (1992) found that solubility of DEHP in blood is significantly better than in water.

A significant number of studies have described the toxicity of DEHP in animals and thus raised concern of its effects on humans. The Environmental Protection Agency (EPA) lists DEHP in the B2 Group of probable human carcinogens. DEHP can enter the body in several ways. First, DEHP leaches from PVC plastic bags into stored blood products at an average rate of 0.25 mg/100ml/day at 4 ºC. This means that during an entire body exchange transfusion of 20-day stored blood, the patient gets about 4 mg/kg of DEHP over a period of a few hours, as documented by Curto and Thomas (1981). Second, the World Health Organization (WHO) (1996) states that it can also leach from plastic wrapping around fat-containing foods such as milk, cheese, and margarine, providing a daily adult dose of about 200 μg. Also, Arcadi and co-workers (1998) found that DEHP can accumulate in neonates via mother's milk. Lastly, patients undergoing kidney dialysis are exposed to approximately 90 mg of DEHP per treatment, according to the WHO (1996). A. Francis (1993) established a reference dose (RfD) for chronic and sub-chronic exposure at 0.02 mg/kg/day; the lowest-observed-adverse-effect level (LOAEL) is 19 mg/kg/day.

Determination techniques explored by Marin and Migallon (2002) such as nuclear magnetic resonance spectrometry, ultraviolet (UV) spectrometry, and UV desorption-mass spectrometry are very useful when the concentrations of phthalates are high. Some chromatographic methods determining trace levels of plasticizers have been used together with a preliminary extraction/concentration step prior to analysis, mostly for environmental applications. However, the underlying theme from a number of studies, including that of Cai, Jiang, and Liu (2003), is that a sensitive, simple, and effective method for the determination of DEHP in environmental and biological samples is still necessary. The developed method for simultaneous determination of DEHP and MEHP by gradient elution HPLC is short and does not require the use of any additives to the mobile phase.

MATERIALS AND METHODS

Instrumentation

The project was conducted on the Agilent 1100 Series Liquid Chromatography System consisting of a vacuum degasser, quarternary pump, automatic autosampler, and variable wavelength UV detector. The Alltech Alltima C_{18}-LL column was 150 mm long with 4.6 mm internal diameter. It was packed with C_{18} bond silica particles of the size 5μ. The UV absorbance maxima of DEHP and MEHP were determined using Hitachi V-2000 UV-Vis Spectrophotometer. All the standard solutions were prepared using the Mettler Toledo AG204 Delta Range analytical balance. All of the pH measurements were performed on a pH meter with a glass electrode (Atlorion PerpHect LogRMeter, Beckman Instruments Combination Electrode). Lastly, the robustness of the method was verified on Dry Lab software.

Chemicals and Reagents

The mobile phase consisted of acetonitrile (ACN) (Fisher Scientific) and deonized (DI) water. Among the other chemicals used in the experiment were sodium phosphate, monobasic (Aldrich Chemical Company, INC.); phosphoric acid 85% (Mallinckrodt); sodium acetate (Aldrich Chemical Company, INC.); acetic acid (J. T. Baker Chemical Co.); tetrabutylammonium bromide (Aldrich Chemical Company, INC.); and cetyltrimethylammonium bromide (Fischer Scientific). The phthalate esters, di(2-ehtylhexyl)phthalate and mono(2-ethylhexyl)phthalate, were obtained from TCI America.

Three stock solutions of phthalate esters were prepared and their exact mass concentration was determined using the analytical balance. First, a stock solution of 277.7 ppm DEHP in 47.37% ACN was prepared. The second stock solution consisted of 425.7 ppm MEHP in 47.37% ACN while the third was a mix of both, DEHP and MEHP containing 151.5 ppm of DEHP and 193.4 ppm of MEHP. Samples of the appropriate concentration were diluted in first set of dilutions based on the following equation:

$$X_{esther}[ppm] = \frac{m_{stock}[g] \times x_{esther}[ppm]}{m_{stock}[g] + m_{solvent}[g]}$$

To minimize human error, a second set of dilutions that were used to determine the detection limit was done by manipulating the injected volumes.

Column Preparation

The C_{18} column was washed before every use with different strength of ACN to assure the elution of any species retained in the column. This was done by eluting the column for 30 minutes with 30%, 50%, and 80% ACN solutions. When buffers were used, the column was washed for an additional 30 minutes after every use with pure 70% ACN solution. In addition, when ion pairing reagent was used the column was washed after every use with 70% ACN for 60 minutes. During the last portion of the project the column was regenerated after every injection for 20 minutes using the initial concentration of ACN (5%) in the gradient elution.

UV Detector Optimization

The absorption spectra of DEHP and MEHP were recorded. Both compounds have similar absorbance with maximum absorbance for MEHP to be at the wavelength 235.2 ηm and for DEHP at 233 ηm. The wavelength of 235 ηm was found to be the optimal condition in this experiment.

RESULTS AND DISCUSSION

Using a high concentration (80%) of ACN in water as a mobile phase, the less polar DEHP was eluted in over 45 minutes following the more polar MEHP. A large capacity factor (k) resulted in a broad peak, which decreased detection sensitivity. The capacity factor is a parameter which indicates how much time the analyte spends in the stationary phase with respect to the mobile phase. MEHP was almost unretained due to its relative higher polarity. Decreasing polarity of the mobile phase helped to delay its retention, but also prolonged the retention of DEHP, which was unsatisfactory due to an even bigger broadness of its peak.

Several selecting parameters were tested to optimize resolution including effect of pH, the role of ion-pairing reagent, combined effects of ion-pairing reagent and pH, and the type of ion-pairing reagent.

Effects of pH of Mobile Phase

In an attempt to control ionization of MEHP, a buffer was introduced into the mobile phase. At low pH, MEHP is expected to be in the non-ionic form, and therefore behave more hydrophobic and retain on the stationary phase for longer time. The change in pH of the mobile phase was not expected to interfere with the retention time of DEHP. Therefore, it was believed that both species would elute in reasonably short time after the flow rate was increased. First, the 80% ACN mobile phase was prepared. After the addition of potassium phosphate buffer a visible precipitation was observed. At 70% ACN no precipitation was found, therefore the mobile phase with buffer of this strength was prepared. The influence of the phosphate buffer on MEHP gave a satisfactory result, increasing its retention time from 1.1 minute to 4.3 minutes with a sharp, tall, and symmetric peak. The retention time for DEHP unexpectedly moved from 16.7 to 42.7 minutes giving very broad peak. The flow of the mobile phase was increased from 1.00 to 1.25 ml/min to speed up the elution of MEHP and DEHP. However, even though the retention time of DEHP decreased to 34.2 minutes, this approach did not lead to a satisfactory result. DEHP was still a late eluter and the retention time for MEHP moved to 3.5 minutes, which didn't leave enough space for the unretained species to increase the flow rate even more and not to have MEHP eluted unretained.

Role of Presence of Ion-Pairing Reagent (IPR)

Tetrabutylammonium bromide (TBAB) was introduced into the mobile phase. MEHP was expected to interact with TBAB loaded on the stationary phase and therefore resulting in longer retention time. Also, a decrease in retention time of DEHP was expected due to its high hydrophobicity. The influence of mobile phase of various concentrations of TBAB was examined up to 20 mM of the TBAB. The retention time for MEHP increased from the original 1.1 minutes to 3.4 minutes and that of DEHP changed only slightly from 16.7 minutes to 17.5 minutes, which was somewhat unexpected.

Combined Effects of pH and TBAB

Since the introduction of a buffer and ion-pairing reagent produced unexpected results, the combination of both was examined for possible a synergistic effect. Mobile phase of 15 mM TBAB and 15 mM acetic acid buffer, adjusted at pH 5.03, was prepared. No synergistic effect was manifested by the results in retention times of both species.

Effects of Type of Ion-Pairing Reagent

Introducing a stronger ion-pairing reagent was a promising idea because the previously used ion-pairing reagent, tetrabutylammonium bromide, has four C-4 aliphatic chains which may not retain strongly on the stationary phase. Therefore, its dispersion interaction was not strong enough to retain TBAB on the column. A mobile phase containing cetyltrimethylammonium bromide (CTMAB) was used together with acetic buffer at pH 4.93. The hydrophobic chains of the CTMAB interact by dispersion force with the C_{16} chain of CTMAB and were therefore retained strongly by the C_{18} column. Since CTMAB is expected to retain strongly on the column and due to the difficulty of washing it form

the stationary phase, a maximum of 10 mM CTMAB was tested. Again, the retention times of both species were not sensitive to this new approach.

Linear Gradient Elution

DEHP and MEHP had a wide retention range, which implies that they were of very different polarities. DEHP, the late eluter, had a large capacity factor which resulted in band broadening. The problem of wide differences in retention between MEHP and DEHP can be solved with the use of gradient elution. Thus, introduction of gradient elution was not only expected to reduce the retention range between DEHP and MEHP, but also to enrich the concentration of impurities in both water and acetonitrile. This is important because these impurities may interfere with the determination of trace amounts of DEHP and MEHP due to baseline noise. The HPLC equipment was programmed to increase the percentage of ACN in the mobile phase from 5 to 100 % over the time of 60 minutes. Figure 1 shows that the retention range decreased significantly, with very sharp, narrow, and symmetrical peaks.

This method was optimized by adjusting the gradient time. The gradient time of 10 minutes showed the most favorable results regarding both the retention times and economic considerations, which are discussed further. MEHP eluted at 6.8 minutes and DEHP at 9.2 minutes (Figure 2), with sharp symmetrical peaks, favorable for good quantification.

Sensitivity Optimization under Gradient Elution

The default setting for ultra violet (UV) detector for maximum sensitivity was 210 nm. A series of samples was studied for the optimal wavelength conditions of the UV detector at 235 nm and 210 nm. As expected, the areas of the five different species detected at 210 nm increase. The area of DEHP increased 2.58 times and the area of MEHP 1.34 times, which justified setting the detector to this wavelength. However, some unidentified species emerged in the chromatograms with a significantly large peak area. These species can be recognized by their retention times. The peak with retention time of 2.4 minutes showed an increase in sensitivity of 4.6 times, which would interfere with the determination of detection limit at this wavelength. The decision to stay on the wavelength of 235 nm was also confirmed by statistical analysis (Table 1). The regression coefficient for DEHP measurements at 210 nm exhibits decreased linearity.

Limit of Quantitation

The smallest concentration of DEHP that could be quantitatively determined with high precision was 3 ppm. The limit of quantitation for MEHP was 2 ppm. The limits of detection for both species were calculated as three times the baseline noise.

SUMMARY

The developed linear gradient HPLC method exhibits a certain simplicity, which makes this method economically favorable and easily transferable among laboratories. The quantitation of both phthalates with elution times less than ten minutes is economical regarding both chemical usage and time-efficiency.

LITERATURE CITED

Albro, P. B. 1986. Absorption, metabolism and excretion of di(2-ethylhexyl)phthalate by rats and mice. Environmental Health Perspectives 65:293-298.

Arcadi, F. A., C. Costa, C. Imperatore, A. Marchese, A. Rapisarda, M. Salemi, G. R. Trimarchi, and G. Costa. 1998. Oral toxicity of bis(2-ethylhexyl)phthalate during pregnancy and suckling in the Long-Evans rat. Food and Chemical Toxicology 36:963-970.

Cai, Y., G. Jiang, J. Liu. 2003. Solid-phase microextraction coupled with high performance liquid chromatography-UV detector for the determination of di-*n*-prophyl-phthalate, di-*iso*-butyl-phthalate, and di-cyclohexyl-phthalate in environmental water samples. DOI: 10.1081/AL-120017698: 389-404.

Curto, K.A. and J. A. Thomas. 1981. Comparative effects of diethylhexyl phthalate or monoethylhexyl phthalate on male mouse and rat reproductive organs. Toxicology and Applied Pharmacology 62:121-125.

Francis, A. 1993. Toxicity summary for Bis(2-ethylhexyl)phthalate. Oak Ridge Reservation Environmental Restoration Program Department of Energy under Contract No. DE-AC05-84OR21400. http://risk.lsd.ornl.gov/tox/profiles/bis_2-ethylhexyl_phthalate_c_V1.shtml. (Access date: Feb 21, 2003).

Hill, S. S., B. R. Shaw, and A. H. B. Wu. 2001. The clinical effects of plasticizers, antioxidants, and other contaminants in medical polyvinylchloride tubing during respiratory and non-respiratory exposure. Clinica Chimica Acta 304:1-8.

Jenke, D. 2003. Organic extractables from packaging materials: Chromatographic methods used for identification and quantification. Encyclopedia of Chromatography DOI:10.1081/E-ECHR-120018659.

Jenke, D. 2003. Chromatographic methods used to identify and quantify organic polymer additives. Encyclopedia of Chromatography DOI:10.1081/E-ECHR-120021147.

Kato, K., T. Yamauchi, K. Higashiyama, and H. Nakazawa. 2003. High throughput analysis of di-(2-ethylhexyl)phthalate metabolites in urine for exposure assessment. DOI: 10.1081/JLC-120022401: 2167-2176.

Koop, C. E., D. R. Juberg, E. P. Benedek, R. W. Brecher, R. P. Cole, M. Corn, V. Covello, T. W. Downes, S. C. Gad, P. Guengerich, J. Higginson, W. H. Konemann, J. C. Lamb, Lundberg, and K. M. Thompson. 1999. A scientific evaluation of health effects of two plasticizers used in medical devices and toys: A report from the American Council on Science and Health. Medscape General Medicine 1(1): 407990.

Lakshmi, S. and A. Jayakrishnan. 1998. Migraton resistant, blood-compatible plasticized polyvinyl chloride for medical and related applications. Artificial Organs 22(3): 222-229.

Lundberg, P., J. Hogberg, and P. Garberg. 1992. Diethylhexyl phthalate. Environmental Health Criteria 131: World Health Organization, Geneva: 1-119.

Marin, M. L. and A. J. Migallon. 2002. Polymer additives: Analysis by chromatographic techniques. Encyclopedia of Chromatography DOI: 10.1081/E-ECHR-120005271: 643-645.

Paris, I., F. Ruggieri, P. Mazzeo, and G. Carlucci. 2003. Simultaneous determination of di-(2-ethylhexyl)phatalate and mono(2-ehtylhexyl)phthalate in human plazma by high-performance liquid chromatography. Analytical Letters 36(12): 2649-2658.

World Health Organization, Geneva. 1996. Guidelines for drinking-water quality, Vol. 2, 2nd ed. Health criteria and other supporting information: 530-540.

Figure 1: Linear Gradient elution. (Gradient time: 20minutes, Gradient range: 5-100% ACN, Flow rate: 1.0 Ml/min) Retention time of MEHP: 15.686 min, Retention time of DEHP: 22.721 min.

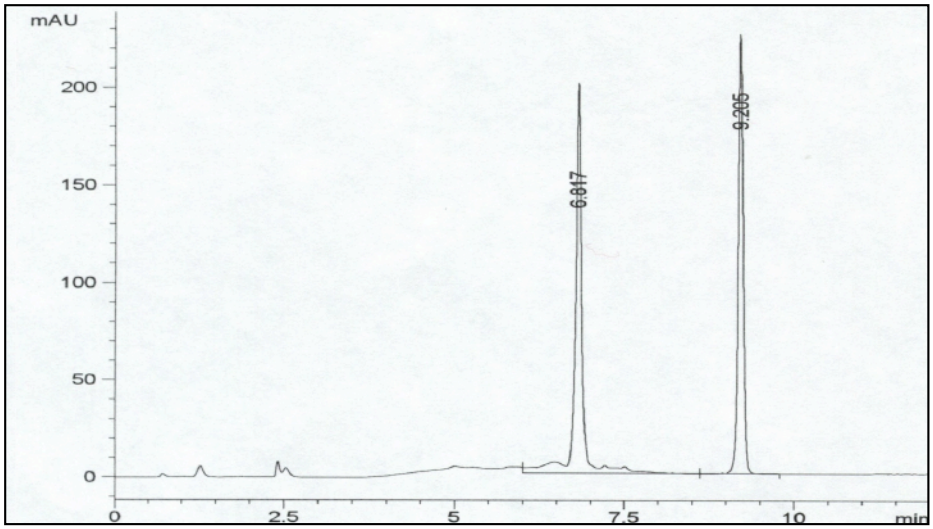

Figure 2: Linear Gradient elution. (Gradient time: 5minutes, Gradient range: 5-100% ACN, Flow rate: 1.0 Ml/min) Retention time of MEHP: 6.817 min, Retention time of DEHP: 9.205 min.

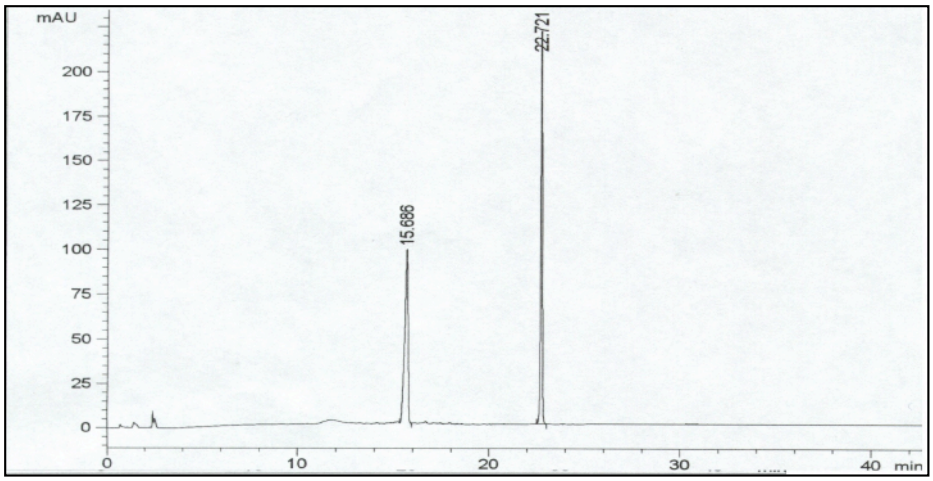

Table 1: The comparison of the regression coefficients for DEHP and MEHP at the wavelengths of 210 ηm and 235 ηm.

	Wavelength: 235ηm	Wavelength: 210ηm
DEHP	31 measurements y = 0.6892*Area – 0.5527 [ppm] R^2 = 0.9938	11 measurements y = 0.023227*Area – 3.0164[ppm] R^2 = 0.8735
MEHP	38 measurements y = 0.0568*Area – 0.8511[ppm] R^2 = 0.9958	12 measurements y = 0.021248*Area – 4.74509[ppm] R^2 = 0.9990