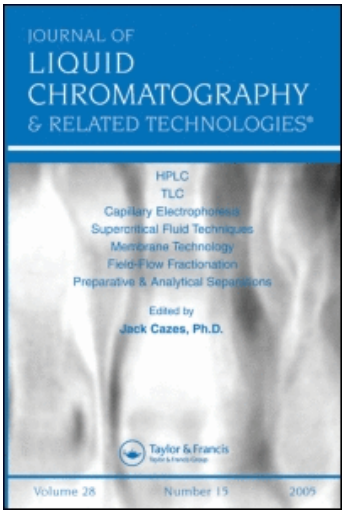

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Unified Reversed-Phase Method for the Determination of Di (2-Ethylhexyl) Phthalate and Its Major Metabolite, Mono (2-Ethylhexyl) Phthalate, in Biological Samples

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Unified Reversed-Phase Method for the Determination of Di (2-Ethylhexyl) Phthalate and Its Major Metabolite, Mono (2-Ethylhexyl) Phthalate, in Biological Samples

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Abstract: A UV detector at 235 nm and an Alltech Altima C_{18} column (150 × 4.6 mm and 5 micron) were used to develop a high performance liquid chromatographic method to determine Di (2-ethylhexyl) phthalate [DEHP] and its metabolite mono (2-ethylhexyl) phthalate [MEHP] in biological samples. A gradient time of 8 min and a gradient range of 60–100% acetonitrile (ACN) at pH 3.0, with a segmented flow rate gradient, were found to be optimum conditions. These conditions resulted in retention times of 4.2 and 7.1 min for MEHP and DEHP, respectively. The estimated limits of detection (LOD) and quantitation (LOQ) for DEHP were 1.37 and 4.8 μg/mL, respectively. For MEHP, LOD, and LOQ were 0.57 and 2.4 μg/mL, respectively. The developed method was applied to determine DEHP and its metabolite MEHP in blood plasma, liver, kidney, brain, and testis samples.

Keywords: DEHP, MEHP, HPLC-UV, Biological samples

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INTRODUCTION

Di (2-ethylhexyl) phthalate, referred to as DEHP (Figure 1), is widely used as a plasticizer in the production of flexible polyvinyl chloride (PVC) products. Health concerns have arisen because of DEHPs widespread use in medical products such as plastic tubing and blood storage bags, as well as in the food industry for various storage products.[1–3] Fatty foods, dairy, fish, meat, and oils are shown to contain the highest level of DEHP due to their low polarity.[4–6] The release of phthalate from pharmaceutical containers, as well as tubing used in medical devices, have been investigated.[7,8] Several studies on the toxicity of phthalates have been reported.[9–12] These studies have found toxicological effects of DEHP on liver, kidney, testes, ovary, and thymus when rats or mice were exposed to DEHP in the diet at various concentrations.

Exposure resulting from in vitro conversion of leached DEHP to MEHP during storage of blood products has shown a conversion rate enhancement by increasing storage time and temperature.[13] Storage at 4°C significantly inhibits conversion of DEHP to MEHP and storing at −30°C prevents its entirely.[13,14] In vivo production of MEHP was also found to depend upon the route of exposure. For example, oral administration results in the greatest conversion of DEHP to MEHP. A much slower conversion rate was observed during transfusion or processing of blood compared to equivalent oral doses.[13]

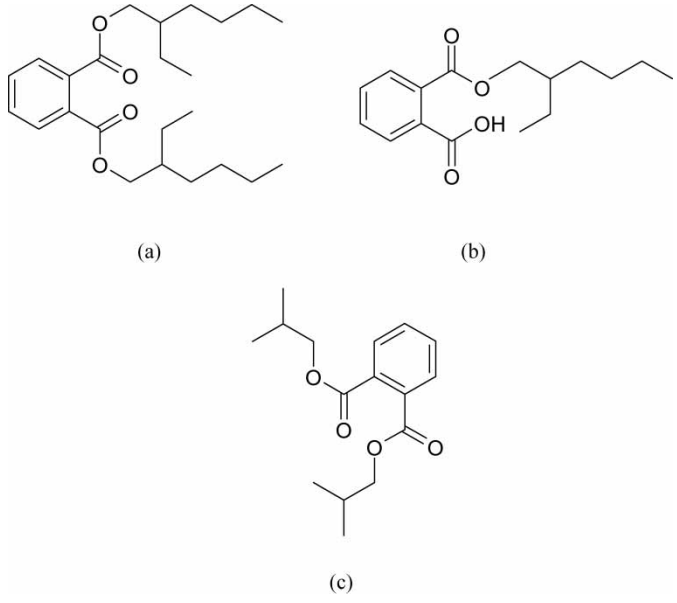

Figure 1. Chemical structures of (a) DEHP, (b) MEHP, and (c) DIBP (internal standard).

A number of analytical methods have been reported for the determination of DEHP in various foods, biological fluids, and tissue samples. These include high performance liquid chromatography,[15–17] as well as gas chromatography.[18–21] Some of these methods are partially validated, and are useful only for DEHP and not for its metabolite. The objective of the present study was to establish a unified stability indicating HPLC method to determine, simultaneously, both DEHP and its oxidative metabolite MEHP in different biological tissues such as rat liver, kidney, testes, brain, and plasma. This study also describes validated extraction procedures for the recovery of DEHP and MEHP from different biological matrices.

EXPERIMENTAL

Chemicals and Reagents

Phthalic acid mono-2-ethylhexyl ester (MEHP), phthalic acid di-2-ethylhexyl ester (DEHP), and phthalic acid diisobutyl ester (DIBP) were purchased from TCI America. HPLC grade acetonitrile (ACN) and methanol (MeOH) were purchased from Fisher Scientific. The water used was purified using a Milli-Q gradient A10 system (Millipore Corp., Milford, MA). All other chemicals and solvents were obtained from commercial sources and were of high purity.

Instruments

The test method was developed on a 1100 Series HPLC system from Agilent Technologies. This system is equipped with a G1314A UV/VIS detector, G1313A autosampler, G1322A degasser, and G1311A quaternary pump. The developed method was validated on a second Agilent 1100 Series HPLC system with a diode array detector.

HPLC separation was performed on an Altima C_{18} column (150×4.6 mm, 5 μm) with a precolumn filter from Alltech Associates Inc. DryLab 2000 software was used to simulate the optimum separation conditions during method development.

A Polytron (Brinkmann Instruments, USA) was used to homogenize tissues and a Sonifier Cell Disruptor (Branson Instruments Inc., USA) was used to further break down the homogenized tissues.

Preparation of Standard Solutions

A stock solution of 23.40 μg/mL DEHP and 43.40 μg/mL MEHP in 100% ACN was prepared by weighing 11.70 mg of DEHP and 21.70 mg of MEHP reference standards in a 500 mL volumetric flask. The reagents were

dissolved in a small portion of 100% ACN then completed to the mark with ACN, and mixed well. A 256 μg/mL stock solution of the internal standard (DIBP) was prepared by weighing 25.60 mg DIBP in a 100 mL volumetric flask, completed to the mark with 100% ACN, and mixed well.

Sample Preparation Procedure

The organs were weighed, then chopped by polytron. Approximately 1 g of the chopped tissue was transferred into a 10 mL reaction vial (for extraction) containing 7.5 mL of 100% ACN, 0.02 mL of 85% phosphoric acid, and 0.3 g of NaCl. After adding 1.5 mL of 256 μg/mL DIBP into the extraction solution as an internal standard, the sample was homogenized for 10 min with the Sonifier Cell Disruptor. The homogenized sample was then vortexed for 5 min and filtrated using an Acrodisc CR 25 mm Syringe Filter, prior to injection into the HPLC system.

For liquid samples, such as plasma and milk, a 200 μL of the sample was transferred into a glass tube, followed by the addition of 1 mL of 100% ACN, 4 μL of 85% phosphoric acid, and 200 μL of the internal standard. The solution was vortexed for one min then filtered to separate proteins and other insoluble substances using the Acrodisc Syringe Filter, prior to injection into the HPLC system.

RESULTS AND DISCUSSION

Method Development

The retention times of DEHP, MEHP, and DIBP from the two linear gradients at 40 and 60 min were used to simulate the optimum separation conditions using DryLab 2000 software. The simulated conditions (60 to 100% ACN at pH 3.0 for 15 min at a flow rate of 1 mL/min) resulted in a run time of 13.9 min [Figure 2(a)]. However, a wide difference in the retention times between DIBP and DEHP indicated the necessity for a segmented gradient.

The steepness of the gradient was increased by decreasing the gradient time from 15 to 10 min without changing the gradient range. No elution of DEHP was observed under these conditions. As a result, the composition of mobile phase was maintained at 100% ACN for 2 min. Although, the elution time decreased from 13.9 to 11.2 min, the difference in retention time between DIBP and DEHP did not change significantly [Figure 2(b)]. Therefore, it was found necessary to increase the steepness of the gradient by decreasing the gradient time from 10 to 5 min, without changing the gradient range. Furthermore, the elution period at 100% ACN was increased from 2 to 4 minutes [Figure (2c)]. The elution time decreased significantly

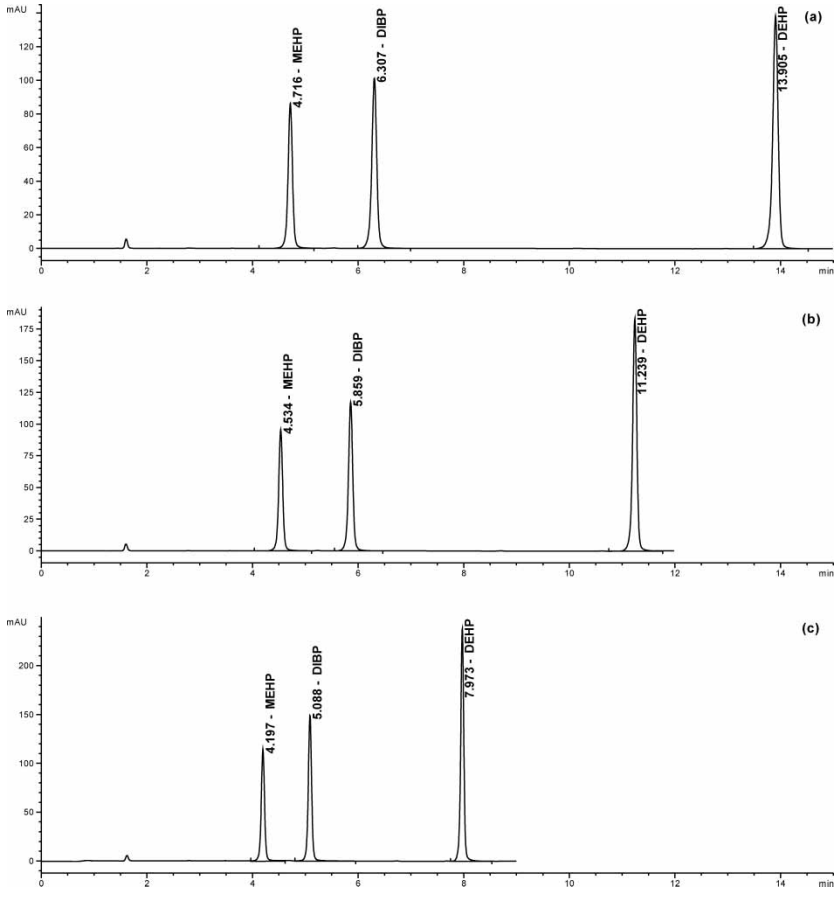

Figure 2. Chromatographic separation of a sample of DEHP, MEHP, and DIBP. Conditions: (a) 60 to 100% ACN for 15 min at a flow rate of 1 mL/min; (b) 60 to 100% ACN for 10 min, and at 100% ACN for 2 min at a flow rate of 1 mL/min; (c) 60 to 100% ACN for 5 min, and at 100% ACN for 4 min at flow rate of 1 mL/min. $NaH_2PO_4 \cdot H_2O$ (25 mM) was used to adjust pH 3.0 in all cases.

from 11.2 to 7.9 min. Meanwhile, the difference in retention times between DIBP and DEHP also improved. At this point, the developed method was considered at its best. Meanwhile, a short separation time and a narrow gradient range are good indications of a short column equilibration time between injections. Also, running the mobile phase at 100% ACN helps in the elution of late eluters (known as 'column killers'), which may be present in the injected samples.

When the developed method was tested on samples extracted from different biological matrices that were spiked with DEHP, MEHP, and

DIBP, it was found necessary to modify the procedure to overcome the interferences of the undesired peaks, especially with liver and kidney samples. A segmented flow rate gradient was introduced in which the flow rate was maintained at 1 mL/min between 60 and 100% ACN at pH 3.0 with a gradient time of 5 min, then increased to 2 mL/min and maintained at that flow rate for 3 min, while keeping solvent strength at 100% ACN. The results of testing the modified method on spiked samples of liver, kidney, and testis and extended to include brain, plasma, and milk samples are shown in Figures 3 and 4. No interferences were observed between sample components and the peaks of interest.

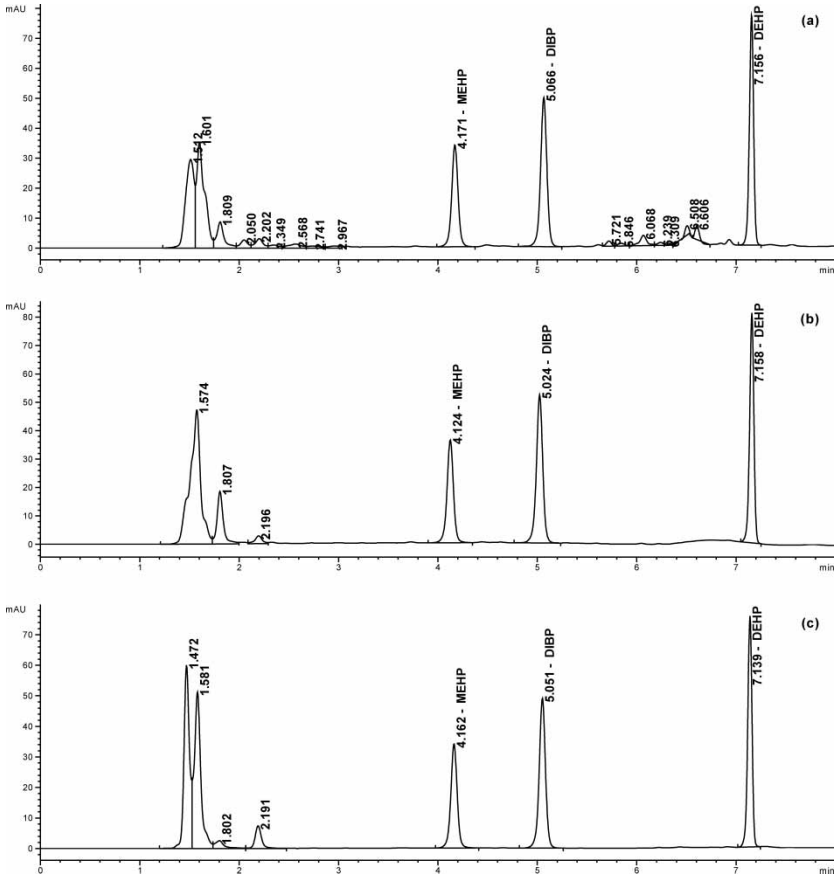

Figure 3. Effect of sample matrix on the separation of DEHP, MEHP, and DIBP; (a) liver; (b) kidney; (c) testis. Condition: 60 to 100% ACN for 5 min at flow rate of 1 mL/min, and at 100% ACN for 3 min at flow rate of 2 mL/min, buffer salt: 25 mM $NaH_2PO_4 \cdot H_2O$, pH 3.0.

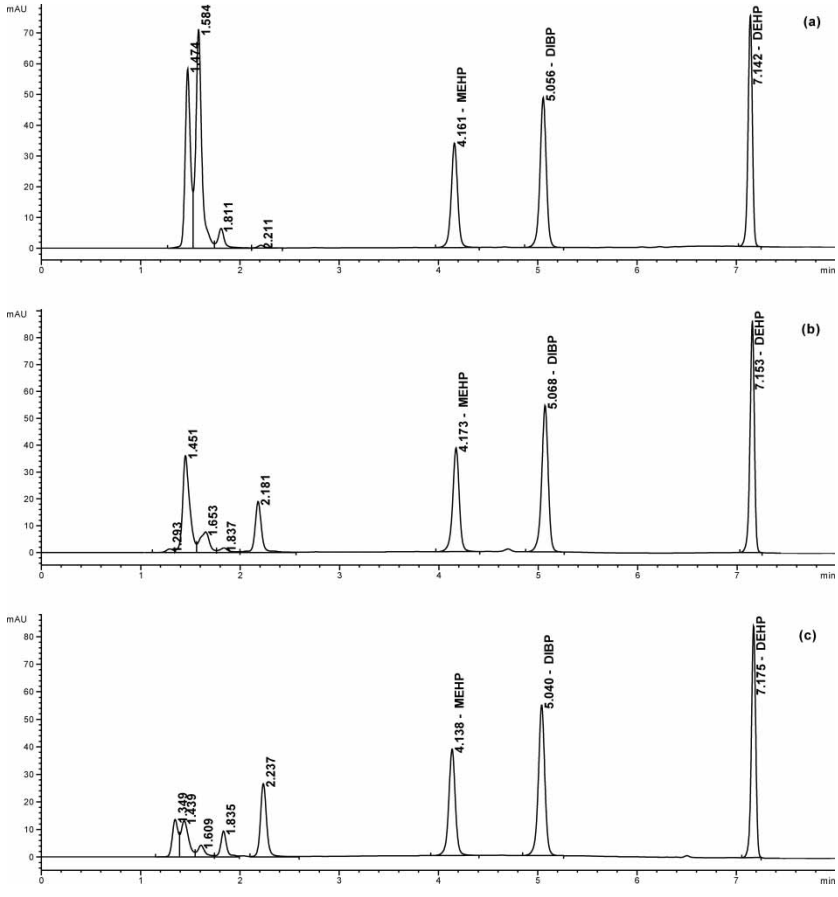

Figure 4. Effect of sample matrix on the separation of DEHP, MEHP, and DIBP; (a) brain; (b) plasma; (c) cow milk. Condition: 60% to 100% ACN for 5 min at flow rate of 1 mL/min, and 100% ACN for 3 min at flow rate of 2 mL/min, buffer salt: 25 mM $NaH_2PO_4 \cdot H_2O$, pH 3.0.

Method Validation

Extraction Procedure

The extraction procedure was validated by testing the percent recovery. A 3 mL standard solution of 237.6 μg/mL DEHP, 77.2 μg/mL MEHP, and 98.0 μg/mL DIBP was added to 1 g of the liver tissue sample. An additional 6 mL of 100% ACN was added during the extraction procedure. This resulted in 99.60% recovery of DEHP and only 89.87% of MEHP. In an attempt to improve recovery of MEHP, acidity of the extracted solution was increased by adding 50 μL of 1 M H_3PO_4. This increased recovery of MEHP to

95.1% without changing that of DEHP. In another attempt to further improve recovery of MEHP, the effect of sodium chloride was tested. A solution of 20 μL concentrated H_3PO_4, 300 mg NaCl, and 6 mL of 100% ACN were added to 1 g of the liver tissue samples. This was followed by the addition of 3 mL of a standard solution of 237.6 μg/mL DEHP, 77.2 μg/mL MEHP, and 98.0 μg/mL DIBP. This resulted in a recovery of more than 99% for both DEHP and MEHP from the liver sample.

The effect of phosphoric acid and sodium chloride on the recovery of DEHP, MEHP, and DIBP from liver, kidney, testis, and brain samples was investigated. These samples were spiked with 79.0 μg/mL DEHP, 25.7 μg/mL MEHP, and 32.6 μg/mL DIBP, and extracted in the presence and absence of phosphoric acid and sodium chloride. The recovery of MEHP improved significantly in the presence of H_3PO_4 and NaCl in all samples studied (Table 1).

The effects of homogenization and vortex shaking times on the percent recoveries of DEHP and MEHP from tissue samples were also tested during validation of the extraction procedure. Homogenization time, using a Sonifier Cell Disruptor, was fixed at 10 min then followed by 5 min of vortex shaking. An Acrodisc CR 25 mm Syringe Filter was used to filter the sample prior to injection into the HPLC system. For plasma samples, 1 mL of plasma was spiked with a 2 mL solution containing 237.6 μg/mL DEHP, 77.2 μg/mL MEHP, and 98 μg/mL DIBP. This was followed by the addition of a 4 mL solution of 20 μL of concentrated H_3PO_4 in 100% ACN prior to extraction and HPLC analysis. It resulted in about 100% recovery of both DEHP and MEHP.

Table 1. Effect of H_3PO_4 and NaCl on percentage recovery of DEHP and MEHP

Sample[a]	% Recovery (DEHP)	% Recovery (MEHP)
Liver sample[b]	100.0	99.2
Kidney sample[b]	99.4	98.7
Testis sample[b]	99.4	99.6
Brain sample[b]	99.8	99.1
Liver sample[c]	100.9	90.2
Kidney sample[c]	99.9	90.9
Testis sample[c]	100.0	95.6
Brain sample[c]	99.9	94.1

[a]Concentration of DEHP, MEHP and DIBP are 79.0, 25.7, and 32.6 μg/mL respectively in each sample.
[b]With H_3PO_4 and NaCl.
[c]Without H_3PO_4 and NaCl.

Table 2. Stability studies of standard solutions of DEHP, MEHP, and DIBP after 48 and 72 h

Conc. DEHP (μg/mL)	Conc. MEHP (μg/mL)	Conc. DIBP (μg/mL)	% Initial value					
			DEHP (48 h)	MEHP (48 h)	DIBP (48 h)	DEHP (72 h)	MEHP (72 h)	DIBP (72 h)
0.00	0.00	26	N/D	N/D	102.1	N/D	N/D	104.0
1.16	1.63	26	75.8	102.2	101.4	78.0	105.0	102.7
2.32	3.26	26	90.9	105.0	101.9	92.4	107.6	103.9
4.64	6.53	26	98.2	102.6	102.9	101.6	105.6	105.8
5.80	8.16	26	97.1	101.0	100.9	98.3	102.7	102.3
6.96	9.79	26	99.4	101.6	101.7	101.4	103.7	103.5
9.28	13.06	26	96.4	98.06	92.6	98.6	100.7	95.2
11.6	16.32	26	98.1	101.6	90.4	99.6	103.2	91.7

Robustness Studies

The stability of the prepared standard solutions was monitored at 48 and 72 h. Peak areas were checked against freshly prepared solutions. The results (Table 2) are expressed in terms of percentage change in peak area and calculated as follow:

$$\% \text{ of initial value} = A_t/A_o \times 100$$

where A_t = peak area at time t; A_o = peak area at time zero.

The percent initial values were lower for DEHP standard solutions at low concentrations after 48, as well as 72 h. Similar results were obtained for the percent initial value of the standard solution in different spiked biological matrices after 48 and 72 h, as shown in Table 3.

Table 3. Stability studies of DEHP and MEHP in biological matrices after 48 h standing

	% Initial value (48 h) (DEHP)	% Initial value (48 h) (MEHP)	% Initial value (72 h) (DEHP)	% Initial value (72 h) (MEHP)
Spiked liver sample	99.9	99.7	99.8	99.9
Spiked kidney sample	100.0	99.9	103.3	100.8
Spiked testis sample	100.2	99.9	99.8	99.3
Spiked brain sample	99.9	99.7	99.9	99.1
Spiked milk sample	97.7	97.9	99.2	99.2

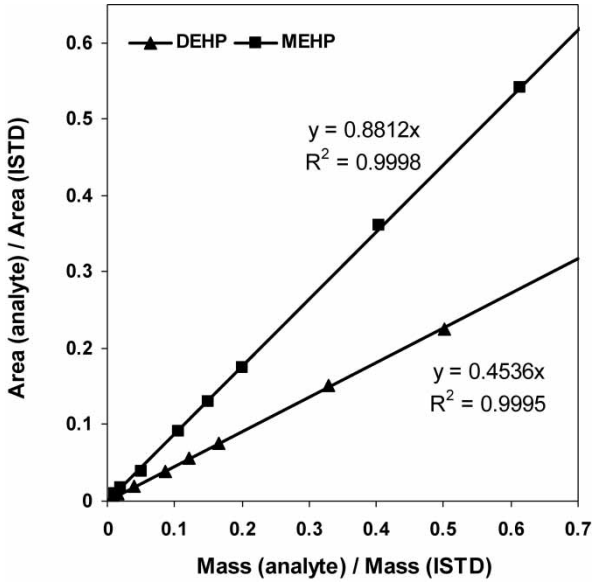

Figure 5. Linearity plot of DEHP and MEHP.

Linearity

Calibration curves for DEHP and MEHP were constructed by plotting the concentration ratio versus peak area ratio for the analyte and the internal standard (Figure 5). These curves showed a zero intercept with correlation coefficients more than 0.999.

The LOD and LOQ for DEHP were also investigated and determined to be 1.37 and 4.8 μg/mL, respectively. For MEHP, the LOD and LOQ were found to be 0.57 and 2.394 μg/mL, respectively. The method appears to be linear in the range of 0.81 μg/mL and 24.78 μg/mL for DEHP and 1 μg/mL and 30.38 μg/mL for MEHP.

CONCLUSIONS

A reversed-phase HPLC method was developed and validated for the determination of DEHP and its metabolite MEHP in different biological samples. A gradient time of 8 min, with a gradient range of 60–100% ACN at pH 3.0 using a segmented flow rate, were found optimum when selecting a C_{18} column (150 × 4.6 nm and 5 micron) and working at 235 nm. Validation parameters tested, confirmed that the developed method is linear, reliable, and with a high degree of recovery of both DEHP and MEHP.

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