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ARTICLE

Development of an Air-assisted Dispersive Liquid-Liquid Microextraction Method as a Valuable Biomonitoring Tool for Exposure Assessment of Phthalates

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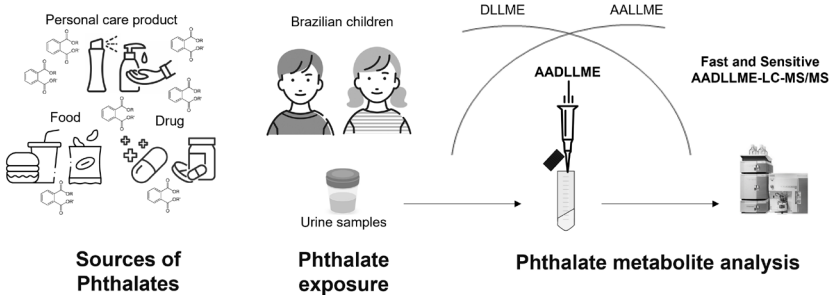

In recent years, the number of epidemiological studies on phthalates that can inform and help update health risk assessments has grown rapidly. Developing reliable and rapid analytical methods for determining phthalate monoesters (m-PAEs) is an important biomonitoring tool for assessing exposure. In this study, a fast and sensitive method was developed to determine 15 m-PAEs in human urine samples as effective biomarkers for exposure assessment. Air-assisted dispersive liquid-liquid microextraction and liquid chromatography coupled to mass spectrometry were used. In order to determine the optimal conditions and model the variables influencing the extraction efficiency, a central composite rotatable design coupled with response surface methodology was used. Under the optimized conditions, the method achieved good linearities ($R > 0.99$), satisfactory intra- and inter-day accuracies (97–111%), and intra- and inter-day precision (RSD < 14%). The proposed procedure allowed the detection of the m-PAEs with limit of detection values between 0.02 and 0.10 ng mL^{-1}, which makes the method sensitive and appropriate for assessing internal exposure to phthalates. The applicability of the proposed procedure was evaluated by screening fifty children's urine from Brazil. High detection frequencies and urinary concentrations of several m-PAEs associated with using personal care products and diet were found.

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INTRODUCTION

In order to fulfill the extensive market demands, high-molecular-mass synthetic organic polymers, commonly known as plastics, are annually produced at a global scale of approximately 380 million tons. These materials have gained widespread use in various aspects of daily life due to their lightweight properties, ability to be shaped, and long-lasting nature. To achieve the softness and durability of plastic products, a specific group of additive compounds, commonly known as “plasticizers”, are incorporated into the manufacturing process. Among these plasticizers, phthalates have been extensively utilized.[1–5]

Phthalates (PAEs) are a group of chemicals derived from 1,2-benzenedicarboxylic acid that is widely used in various commercial applications. They can be divided into high-molecular-mass PAEs, which act as plasticizers to increase product flexibility and durability, and low-molecular-mass PAEs, used for color and fragrance retention or to provide a film or gloss. These chemicals are extensively present in everyday items like food packaging, toys, medical devices, and personal care products. Due to their non-chemical binding to polymer chains, phthalates are released into the environment, becoming ubiquitous environmental contaminants. Consequently, widespread exposure to different phthalates has been reported among the general population.[2,6–16]

Exposure to phthalates has been associated with various health issues, including reproductive and endocrine disorders. As a result, it is crucial to conduct biomonitoring to assess human exposure to phthalates. Currently, the most widely accepted method for this assessment in epidemiology studies is the measurement of m-PAEs in urine. Although direct analysis without any extraction or purification step has been documented as a time-efficient screening method for determining m-PAEs, it is necessary to perform sample preparation procedures to eliminate endogenous compounds present in urine. Failure to remove these compounds can lead to column clogging and interfere with analyte ionization in the electrospray ionization source.[11,12,17–23]

In human biomonitoring studies, sample preparation is one of the most essential steps to determine m-PAEs. The two most common techniques for analyzing m-PAEs are liquid-liquid extraction and solid-phase extraction. However, the consumption of time, solvent, and labor intensiveness make these approaches less attractive for large-scale human biomonitoring studies. Consequently, alternative procedures combining rapidity, simplicity, a reduced volume of samples, and the consumption of toxic reagents are in high demand.[9,11,13,24–28] Thus, microextraction techniques are becoming excellent alternatives for the analysis of these metabolites in urine samples.[29–34]

Among these techniques, dispersive liquid-liquid microextraction (DLLME) and air-assisted liquid-liquid microextraction (AALLME) have been increasingly used for the extraction of organic compounds in biological samples owing to their advantages of low detection limits, low consumption of organic solvent, short extraction time, and simple operation.[35–38] In the present study, the feasibility of these two liquid-phase microextraction methods (AALLME and DLLME) was simultaneously used as a new microextraction procedure (AADLLME) for the fast and sensitive determination of 15 m-PAEs in urine samples. The Response Surface Methodology - Central Composite Rotational Design (CCRD-RSM) was applied to optimize the parameters affecting the AADLLME. The proposed method has been validated and applied to determine internal exposure to phthalates by measuring specific metabolites in urine samples collected from 50 Brazilian children (6–14 years old).

MATERIALS AND METHODS

Materials and reagents

A method for determining the urinary level of 15 phthalates metabolites encompassing a total of 11 parent phthalates was proposed in the current study. The analytical standards for 15 phthalates metabolites and 14 internal standards were purchased from multiple suppliers (Table S1). Specific information concerning

m-PAEs analyzed, their respective parent compounds, chemical names, and their abbreviations are presented in Table S1. Phthalate metabolite standards were dissolved in methanol to create standard stock solutions with a concentration of 1000 ng mL^{-1}, and working solutions for use in method development and analysis were prepared weekly by the appropriate dilution of the stock solution by water/methanol solution to the required concentration.

All solvents [methanol (MeOH), 1,2-dichloroethane (DCE), and trichloromethane (TCE), dichloromethane (DCM), acetone (ACE) and acetonitrile (ACN)] were of HPLC grade and were obtained from JT Baker® (Phillipsburg, NJ, USA) and Sigma-Aldrich® (St. Louis, MO, USA). Ultrapure water (18.2 MΩ·cm resistivity) was obtained from a Milli-Q water purification system (Millipore®, Bedford, MA, USA). The reagents (analytical grade) employed for preparing synthetic urine were obtained from Sigma-Aldrich® (St. Louis, MO, USA). Detailed information regarding the preparation of synthetic urine is presented elsewhere.[36–39]

Instrumental apparatus and analysis conditions

For the analysis of phthalates metabolites and their corresponding isotope-labeled surrogates, chromatographic separation, identification, and quantification of the targets was carried out using a Thermo Scientific liquid chromatography-tandem mass spectrometry system equipped with a quaternary pump (Accela 600) and an automatic sampler coupled with a triple quadrupole mass spectrometer detector (TSQ Quantum Access Max) with source ionization by electrospray (Thermo Fisher Scientific®, USA) in negative mode. Our previous work has detailed the instrumental parameters for mPAEs analysis.[38] The mPAEs-specific *m/z* transitions of multiple reaction monitoring and optimized MS/MS parameters acquired by direct infusion of each metabolite using an in-built syringe pump are shown in Table S2.

Urine samples and pretreatment

Fifty urine samples were randomly selected for a collaborative project between the Brazilian Ministry of Health and the University of São Paulo. The project aimed to establish reference values for organic and inorganic contaminant exposure in elementary school children (6–14 years old). All urine specimens were kept at -80 °C until further use. Further information on the urine samples can be found elsewhere.[40,41] Before the microextraction procedure, the urine samples were subjected to enzymatic hydrolysis. For this purpose, the samples were thawed and mixed at room temperature and then subjected to centrifugation at 2000 *g* for 10 minutes. Aliquots of 2.5 mL of the urine samples were transferred to a polypropylene tube with a conical bottom, and 20 µL of the freshly prepared internal standard solution, 500 µL of ammonium acetate buffer (1 mol L^{-1}, pH 5.5), and 20 µL of β-Glucuronidase K12 from *E. coli*, arylsulfatase-free (Roche Diagnostics®, Mannheim, Germany), were added. Afterward, the urine samples were gently mixed and incubated for 3 h at 37 °C for enzymatic hydrolyses of the conjugates.

Sample preparation

The enzymatically treated samples were transferred to conical-bottom polypropylene tubes, diluted to 5 mL with water, and gently mixed. The pH of the sample solution was adjusted to 2 by adding hydrochloric acid solution (0.1 mol L^{-1}). At the beginning of the extraction process, a mixture containing 1200 µL of ACN (dispersive solvent) and 900 µL of DCE (extraction solvent) was rapidly injected into the sample solution using a glass syringe (2.5 mL). For the second step of the extraction process, the 10 mL glass syringe was inserted into the mixture, and five extraction cycles of suction and injection were performed into the tube for the dispersion. After five extraction cycles, a second cloudy solution was obtained. The cloudy solution was then centrifuged at 2500 g for 20 min at 20 ºC, and the extraction solvent (sediment layer) was collected and transferred to a 2.0 mL tube. The sedimented phase was dried, being the residue redissolved in a mixture of 200 µL of methanol-deionized water (1:1 v/v) and mixed for 10 s. Finally, 10 µL was injected into the LC-MS/MS system for the analysis.

Experimental design and data analysis

Data analysis was performed using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA), and GraphPad Prism v.9.1.1 (GraphPad Software, San Diego, CA, USA). Quantitative experimental data of the phthalate extraction efficiency (EE%) obtained during the optimization step were screened for normal distribution using the Shapiro–Wilk test. The Shapiro-Wilk test confirmed that samples were normally distributed ($p > 0.05$). Consequently, a Tukey's multiple comparisons test was used to compare the EE% between the extraction/dispersive solvent pairs groups, with significance set at $p < 0.05$. As the data followed a normal distribution, we used bivariate correlation of Pearson to evaluate the associations between the results of EE%. Strong significant positive correlations were found among the EE% of m-PAEs. Therefore, the response value was set as the geometric mean of the EE% of the studied compounds. Descriptive statistics, such as geometric mean, median, minimum, and maximum, are expressed as volume-based concentration (ng mL^{-1}). Concentrations below the LOD were replaced with LOD/√2.

To establish the correlation between dependent and independent variables, Response Surface Methodology (RSM) was employed. Central Composite Rotatable Design (CCRD) was utilized to obtain the maximum amount of information about the process while conducting a minimal number of experiments. This approach aimed to identify the relationship between the response and the variables and determine the optimal extraction conditions for m-PAEs using AADLLME.[42] Based on previous studies,[36–38] four factors were studied: the volume of extraction solvent (X1), the volumes of dispersive solvent (X2), the number of aspiration/dispersion cycles (X3), and the ionic strength (X4). A 24 CCRD with triplicates of the central point was employed, totaling 27 runs (Table S3). The experiments were conducted randomly to minimize uncertain variability that could potentially influence the response outcomes due to extraneous factors. The experimental data were fitted to a second-order polynomial model (Equation 1).

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k} \sum_{j=1}^{k} \beta_{ij} X_i X_j \qquad \text{Equation 1}$$

Where β_0, β_i, β_{ii}, and β_{ij} represent regression coefficients, X_i, and X_j are the independent variables, Y is the response (geometric mean of EE%), and k is the number of the independent variables. In turn, Equation 2 determines the number of the required experiments (N), including factorial points (2^n), axial points ($2n$), and central points (N_0).

$$Y = 2^n + 2n + N_0 \qquad \text{Equation 2}$$

Three-dimensional response surface plots were utilized to estimate the optimal extraction conditions. To assess the quality of fit of the polynomial model, coefficients of determination (R^2), adjusted coefficients of determination (R^2adj), and predicted coefficients of determination (R^2pred) were obtained. Analysis of variance (ANOVA) provided the lack-of-fit and F values, which were used to evaluate the significance and adequacy of the model. All fitting procedures, coefficient estimates, and statistical analyses were conducted using the Design-Expert software.

RESULTS AND DISCUSSION

Optimization of extraction conditions

The AADLLME conditions were assessed and optimized by both a one-factor-at-a-time (OFAT) approach and a multi-factor method with a CCRD to get the best EE% of m-PAEs from human urine samples. The main AADLLME experimental factor, the type of the extraction and dispersive solvents, were first investigated by the OFAT method (experiments were performed in triplicate), while the volume of the extraction and dispersive solvents, the numbers of extraction cycles, and ionic strength, were investigated and optimized by RSM with a CCRD.

The EE% was calculated by comparing the response (peak area) between the synthetic urine (blank matrix) (2.5 mL) spiked with 10 ng mL^{-1} of each mPAE and in a post-extraction matrix spiked at the same concentration through Equation 3. Since there is no human urine exempt from m-PAEs, we employed synthetic urine as a blank matrix.

$$\frac{Peak\ area\ of\ the\ pre-extraction\ fortificated\ matrix}{Peak\ area\ of\ the\ post-extraction\ fortificated\ matrix} \times 100 \qquad \text{Equation 3}$$

The pH of the sample is another commonly optimized parameter in liquid-phase extraction methods. For ionizable analytes, neutral forms tend to be more efficiently extracted into the organic phase. Thus, the sample pH plays a crucial role in the extraction process. In this study, in order to achieve full protonation of the -COOH group in m-PAEs, all test samples were subjected to acidification using hydrochloric acid until reaching a pH value of 2, taking into consideration the pKa of the m-PAEs (pKa, 3.4 – 3.6).[13]

Selection of the solvent pair

A key aspect of microextraction procedures involves dispersing the extraction solvent into the aqueous sample. It is crucial that both the extraction solvent and the aqueous sample are miscible with the appropriate dispersive solvent. Additionally, the dispersive solvent should effectively disperse the extraction solvent through the aqueous solution, creating a cloudy solution to enhance the contact area between the two phases and accelerate the extraction process.[43] Consequently, selecting the dispersive-extraction solvent pair becomes one of the most significant factors in extraction optimization. To identify the optimal solvent pair, all possible combinations between the extraction and dispersive solvents were thoroughly examined.

According to the EE% (geometric mean of all m-PAEs extraction efficiency) shown in Figure 1, the best condition to extract m-PAEs was the combination of DCE and ACN as extraction and dispersive solvent, respectively. Moreover, Tukey's multiple comparisons test was also used to assess the significance of the differences between the groups of extraction and dispersive solvent pairs. The results showed significant differences between all groups except for the following combination, TCE:ACT *vs*. DCE: MeOH. Due to its higher EE%, the combination of DEC and ACN was chosen as the pair of extraction and dispersive solvent for the subsequent optimization experiments.

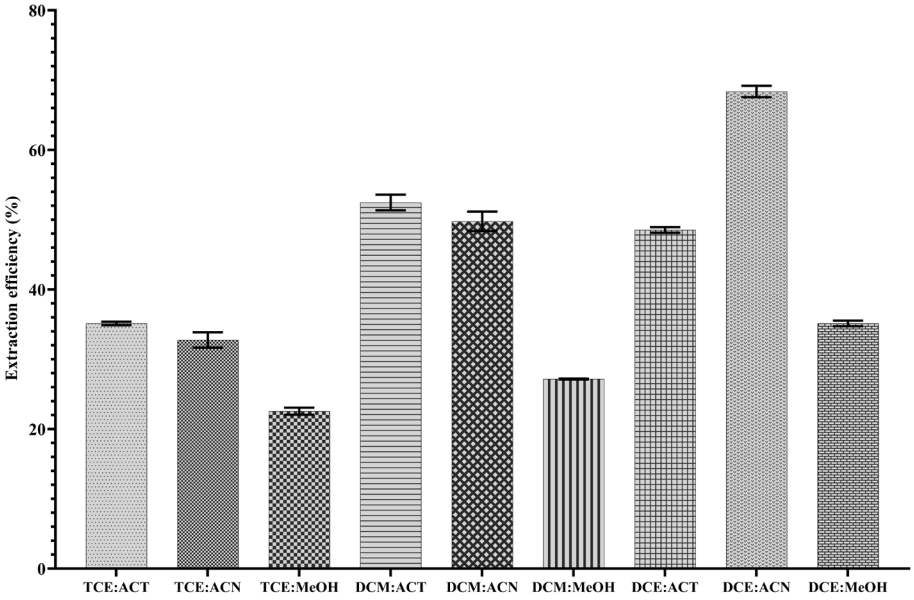

Figure 1. The effect of extraction/dispersive pair solvent type on extraction of phthalate monoesters from urine by AADLLME.

Model adjustment and statistical analysis

In order to generate second-order polynomial models in RSM, the current study uses a CCRD to evaluate the quadratic response surface and detect interactions between the tested parameters. The effects of four important parameters, including the volume of extraction solvent (X_1), the volume of dispersive solvent (X_2), the number of aspiration/dispersion cycles (X_3), and the ionic strength (X_4), were assessed to maximize the EE% of m-PAEs from urine samples. The coded and actual values of the parameters and the response (*Y*) are shown in Table S3. A predictive quadratic polynomial model was established using multiple regression analysis on the experimental data, and the following equation (coded) was used to represent the relationship between the dependent variables (response) and the independent variables (factor) (Equation 4).

$$Y_{(response)} = 81.7 + 1.88X_1 - 3.45X_1^2 + 8.79X_2 - 7.45X_2^2 + 3.37X_3 - 5.32X_3^2 + 2.13X_4 - 3.95X_4^2 - 2.44X_1X_2 + 0.56X_1X_3 - 0.69X_1X_4 + 0.94X_2x_3 - 1.56X_2X_4 + 2.19X_3X_4 \quad \text{Equation 4}$$

By using ANOVA on the experimental data, the significance and fitness of the quadratic model were statistically confirmed. Different statistical variables are presented in the ANOVA (Table I), including degrees of freedom (DF), F-value, p-value, the sum of squares, and mean square. The value of *p* and F-test were used in this analysis. In simple terms, the p-values below and above 0.05 for the fitted model and the lack-of-fit perspective suggest that the second-order model acquired is statistically significant with a confidence level of 95%.

The quadratic fitting model displayed statistical significance, supported by the small p-value (<0.0001) and the substantial F-value (20.07). Additionally, the lack-of-fit test (p-value: 0.323; F-value: 2.46) confirmed the model's appropriateness for data representation at a 95% confidence level. Notably, terms within the model, including both linear and quadratic components, exhibited significant effects on EE% with p-values below 0.05. However, only the interactions X_1X_2 and X_3X_4 demonstrated statistical significance (Table I). The presence of these significant statistical interactions between two independent variables justifies the utilization of RSM as an optimization tool. Traditional one-factor-at-a-time approaches would be inadequate for evaluating such terms, as they would require a larger number of tests, leading to increased costs and time consumption.

The data closely aligned with the second-order polynomial equation, according to the estimated values for the R^2 (0.9590), R^2adj (0.9112), and R^2pred (0.7748), while the pertinent quadratic model had acceptable accuracy and reliability. Adequate precision compares the range of the predicted values at the design points to the average prediction error, and a ratio greater than 4 indicates adequate model discrimination. For the model obtained, it was equal to 16.95, which implies that this model can be used to navigate the design space. In addition, the agreement between predicted and actual values (illustrated in Figure 2), where residuals are randomly distributed, is sufficient to indicate that the model is adequate. These results imply that models can accurately predict the effects of all variables on the EE% of m-PAEs.

Table I. Analysis of variance for response surface quadratic model used for optimization of the extraction of phthalate monoesters

	SS[a]	df[b]	MS[c]	F	p
Model	3950.4	14	282.2	20.1	< 0.0001
A-Extraction Volume	84.4	1	84.4	6.00	0.0306
B-Dispersive Volume	1855.0	1	1855.0	131.9	< 0.0001
C-Cycles	273.4	1	273.4	19.4	0.0009

(continues on the next page)

Table I. Analysis of variance for response surface quadratic model used for optimization of the extraction of phthalate monoesters (continuation)

	SS[a]	df[b]	MS[c]	F	p
D-Ionic strength	108.4	1	108.4	7.7	0.0168
AB	95.1	1	95.1	6.8	0.0232
AC	5.1	1	5.1	0.4	0.5597
AD	7.6	1	7.6	0.6	0.4774
BC	14.1	1	14.1	1.0	0.3370
BD	39.1	1	39.6	2.8	0.1214
CD	76.6	1	76.6	5.4	0.0378
A^2	253.6	1	253.6	18.0	0.0011
B^2	1183.4	1	1183.4	84.2	< 0.0001
C^2	604.5	1	604.5	43.0	< 0.0001
D^2	332.5	1	332.5	23.6	0.0004
Residual	168.8	12	14.1		
Lack of Fit	156.1	10	15.6	2.5	0.3230
Pure Error	12.7	2	6.3		
Cor Total	4119.2	26			

[a]sum of squares; [b]degree of freedom; [c]Mean square

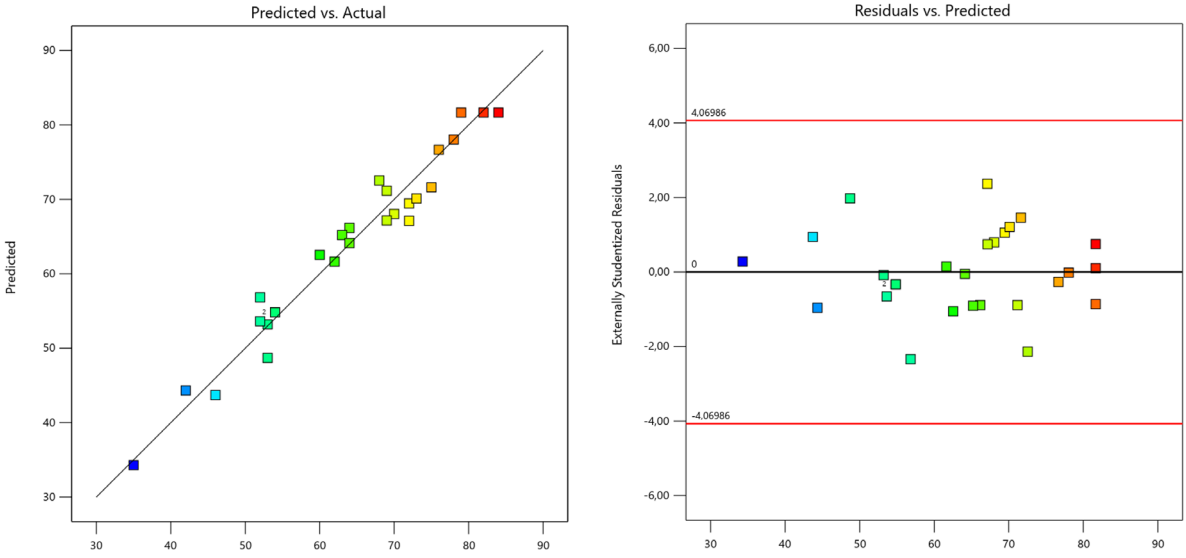

Figure 2. Plot of predicted values versus observed values and plot of residuals versus predicted values for EE% of phthalate monoesters.

Three-dimensional response surface

The development of mathematical models for assessing variables and how they interact with extraction recoveries is frequently done using RSM, a useful statistical technique for optimizing extraction conditions.[44] Figure 3 shows the 3D response surface graphs reflected by the above quadratic polynomial model. These graphs show the responses to two experimental factors, while the others are maintained at their central point conditions. The curvature in the 3D plots (Figure 3) shows these interactions, indicating that the maximum EE% was reached around the center points. Based on the analysis of the 3D graphs, as the volume of extraction solvent and dispersive solvent increased, the EE% initially rose but eventually reached a point of stability or even a decrease. By increasing the extraction and dispersive solvent volumes, the solubility of the m-PAEs improved in the aqueous phase, reducing the EE%. Additionally, comparable trends in variation were identified with the increase in both the number of cycles of extraction and the ionic strength. Adding neutral salt into the aqueous samples, such as urine, can enhance EE% through the salting-out effect, which reduces the analyte's solubility in the aqueous phase and facilitates its transfer to the extraction solvent (organic phase). However, this process might lead to a decrease in the diffusion rates of the extracted compounds into the organic drops due to alterations in the physical properties of the Nernst diffusion film.[37] Additionally, performing an elevated number of aspiration/dispersion cycles may accelerate the dissolution of the extraction solvent in the aqueous phase, reducing the volume of the organic/extraction phase.

The optimization modeling suggested the following optimum experimental conditions for different extraction variables: pH of 2.0, 900 µL of DCE as extraction solvent, 1200 µL of CAN as a dispersive solvent, 5 extraction cycles (aspiration/dispersion), and addition of 11% of sodium chloride. To validate the predicted model from the optimization experiment, the extraction of m-PAEs from synthetic urine samples was conducted under optimal conditions in triplicate. The average EE% of m-PAEs (82%) was in good agreement with the value of the predicted EE% (84%). Statistical analysis using the t-test ($p < 0.05$) revealed no significant difference between the predicted and experimental results. The close agreement between the predicted and experimental values obtained under optimal conditions demonstrates the model's suitability and reliability for achieving high recoveries of m-PAEs from urine samples.

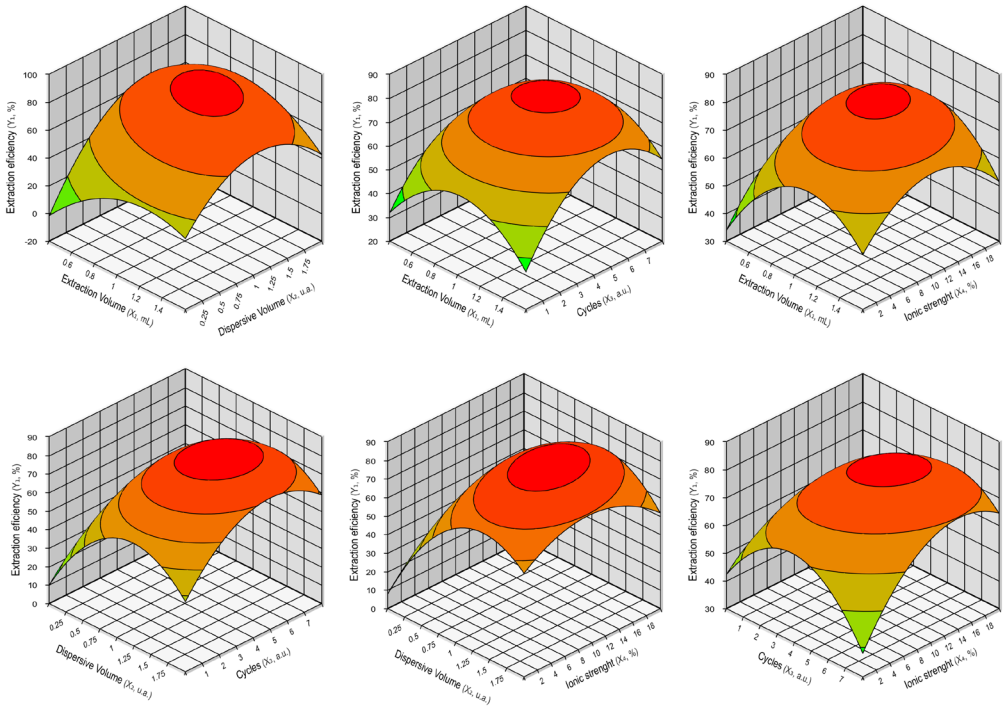

Figure 3. Three-dimensional response surface of the EE% of phthalate monoesters.

Analytical figures of merit

The feasibility of the proposed procedure was evaluated using synthetic urine samples designed to simulate the composition of uncontaminated human urine samples, given the unavailability of blank urine samples.[36–39] To evaluate the analytical performance of the proposed method, a series of parameters of the analysis, including linearity, the LOD, LOQ, precision (%RSD), and recovery, were validated under optimal conditions. The analytical performance results are given in Table II.

Calibration curves were obtained by least-squares linear regression analysis of the ratio between the analytes and the internal standard (with concentration fixed at 10 ng mL^{-1}) peak areas, using seven concentration levels in triplicate ranging from 1.00 to 20.0 ng mL^{-1}. All analytical curves had correlation coefficients above 0.99, showing suitable adjustments to the selected mathematical model. However, these correlation coefficient values alone do not guarantee the linearity of the method. Therefore, linearity was also verified by the following parameters: residual value of replicates and lack-of-fit test significance. The residual value between experimental and predicted responses was lower than 20%, and the lack-of-fit model indicated that the equations of calibration curves (within the concentration range) did not show a lack of fit for the residual variance of the experimental data collected since the calculated p-values were not statistically significant for the models of linear regression of the m-PAEs.

The sensitivity of the methodology was ascertained through the LODs and LOQs for a concentration having an S/N of 3/1 and 10/1, respectively. The LODs and LOQs were estimated by visually examining chromatograms from blank synthetic urine containing decreasing concentrations of the studied compounds. LODs and LOQs of the method ranged from 0.02 to 0.10 ng mL^{-1} and 0.07–0.33 ng mL^{-1} in synthetic urine, respectively. The LOQs and LODs estimated in the present study (Table II) are appropriate for quantifying m-PAEs in human urine samples as demonstrated in human biomonitoring studies.[11,17,18,21,24,27,30,41,45–48]

To evaluate the proposed method's repeatability and accuracy, the precision (RSD%) and recovery (%) were assessed by extracting and measuring synthetic urine samples with three spiked concentrations (1.0, 10.0, and 20.0 ng mL^{-1}) on one day (for intra-day precision) and three different days (n = 6) (for inter-day precision). The relative standard deviation values for intra-day and inter-day precision were in the ranges of 1–14% and 2–13%, respectively. Recoveries were observed in the ranges of 97–111% and 96–112%, respectively. Therefore, the proposed method showed excellent repeatability/reproducibility and accuracy for the analysis of m-PAEs in urine samples.

Table II. Analytical performance of the proposed method for measuring phthalate monoesters in human urine samples

Phthalate monoesters	R	Spiked (ng mL^{-1})	Intra-day		Inter-day		LOD (ng mL^{-1})	LOQ (ng mL-1)
			Recovery (%)	RSD%	Recovery (%)	RSD%		
mMP	0.991	1.0	98	3	104	9	0.02	0.07
		10.0	105	9	108	4		
		20.0	106	9	97	5		
mEP	0.994	1.0	97	5	102	2	0.05	0.17
		10.0	99	2	107	13		
		20.0	104	11	96	9		
mIPrP	0.993	1.0	101	2	98	8	0.02	0.07
		10.0	107	9	107	10		
		20.0	105	4	104	12		
mPrP	0.996	1.0	104	9	99	8	0.02	0.07
		10.0	103	14	112	8		
		20.0	97	2	101	6		

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Table II. Analytical performance of the proposed method for measuring phthalate monoesters in human urine samples (continuation)

Phthalate monoesters	R	Spiked (ng mL^{-1})	Intra-day		Inter-day		LOD (ng mL^{-1})	LOQ (ng mL-1)
			Recovery (%)	RSD%	Recovery (%)	RSD%		
mBuP	0.993	1.0	104	1	105	5	0.05	0.17
		10.0	101	3	109	2		
		20.0	101	9	96	6		
mIPeP	0.997	1.0	101	4	109	13	0.05	0.17
		10.0	97	6	98	6		
		20.0	103	9	101	6		
mCHP	0.996	1.0	109	9	109	10	0.02	0.07
		10.0	100	12	106	13		
		20.0	107	2	104	3		
mCPP	0.995	1.0	105	11	105	11	0.03	0.10
		10.0	97	2	102	4		
		20.0	105	1	108	7		
mBzP	0.997	1.0	98	9	111	8	0.03	0.10
		10.0	109	12	109	13		
		20.0	101	12	107	3		
mEHP	0.991	1.0	106	2	107	4	0.05	0.17
		10.0	104	12	106	10		
		20.0	106	1	100	12		
mEOHP	0.992	1.0	107	13	101	8	0.05	0.17
		10.0	104	1	99	3		
		20.0	99	5	100	2		
mEHHP	0.992	1.0	98	13	104	11	0.03	0.10
		10.0	106	9	109	11		
		20.0	99	5	99	3		
mCMHP	0.994	1.0	101	3	107	5	0.03	0.10
		10.0	97	7	98	11		
		20.0	103	12	102	11		
mECPP	0.995	1.0	109	14	111	2	0.03	0.10
		10.0	101	6	101	13		
		20.0	100	2	111	13		
mCIOP	0.996	1.0	106	5	110	2	0.10	0.33
		10.0	101	5	105	10		
		20.0	108	5	98	2		

Phthalate monoesters determination in real urine samples

The applicability of the validated method was demonstrated by analyzing 50 urine samples taken from Brazilian children participating in a monitoring program.[40,41] The measured (geometric mean, median, minimum, and maximum) volume-based concentrations and detection frequencies of 15 m-PAEs are given in Table III. Among the 15 m-PAEs, mEP, mMP, mBuP, mIPeP, mCPP, and metabolites of di-(2-ethylhexyl) phthalate (mEHP, mEHHP, mECPP, mCMHP, and mEOHP) were found in all samples, which indicated

the ubiquitous exposure to these compounds for Brazilian children. The detection rates of mPrP, mCIOP, mCHP, mBzP, and mIPrP were 88%, 86%, 72.0%, 64%, and 40%, respectively. The frequent detection and high concentrations of these m-PAEs found in urine samples from Brazil, when compared to those of other countries, is possibly linked to extensive exposure to phthalate diesters via personal care products (mEP, mBuP, and mMP) and diet (∑5DEHP). The urine samples showed relatively high concentrations of dibutyl- (mBuP=82.7 ng mL^{-1}), diethyl- (mEP=65.4 ng mL^{-1}), and di-(2-ethylhexyl)-phthalate monoesters(∑, mEOHP, mEHHP, mCMHP, and mECPP = 142 ng mL^{-1}), when compared to existing data reported in the scientific literature.[7,11,13,20,26] The median concentrations of mIPrP and mPrP, metabolites of diisopropyl phthalate and di-n-propyl phthalate, were 0.45 and 0.89 ng mL^{-1}, respectively.

Furthermore, a monoester metabolite of diisopentyl phthalate (DiPeP), mIPeP, were detected in all samples, with concentrations that ranged from 0.09 to 139 ng mL^{-1} (median: 4.36 ng mL^{-1}). Our previous studies have demonstrated ubiquitous exposure of Brazilian to DiPeP, one of the most potent antiandrogenic phthalates. However, this exposure has been observed only sporadically or not at all in studies conducted in different countries.[11,17,18,21,24,27,30,41,45–51] Overall, the current findings are consistent with the results for other populations previously studied using traditional analytical methods.[11,17,18,21,24,27,30,41,45–48]

Table III. Urinary levels of phthalate monoesters (ng mL^{-1}) in children from Brazil (n=50)

Phthalate monoester	Detection rate (%)	Geometric mean	Median	Minimum	Maximum
mMP	100	8.92	9.50	0.82	89.2
mEP	100	73.5	65.4	1.05	3568
mIPrP	40	0.36	0.45	0.05	7.57
mPrP	88	0.86	0.89	0.12	18.1
mBuP	100	61.0	82.7	2.01	2420
mIPeP	100	3.84	4.36	0.09	139
mCHP	72	1.23	1.00	0.06	85.0
mCPP	100	1.89	2.05	0.29	40.7
mBzP	64	2.95	2.35	0.34	47.9
mEHP	100	19.3	24.2	1.90	452
mEOHP	100	15.3	17.5	0.87	1036
mEHHP	100	21.7	24.9	0.74	1230
mCMHP	100	18.5	18.1	0.86	995
mECPP	100	59.1	57.3	1.75	1953
mCIOP	86	3.10	2.50	0.09	278

Comparison of AADLLME-LC-MS/MS with other methods for m-PAEs determination in urine samples

A comparison of the proposed analytical method with others previously published, employing different sample preparation and instrumental techniques for the simultaneous determination of m-PAEs in urine samples, is summarized in Table IV. Our proposed method generally offers some improvements to the previously published methodologies for mPAEs determination in human urine samples.[9,29,31,32,47,52–54] For example, sample preparation requires almost the same time compared to other microextraction methods

but much less time compared with classical methodologies, such as LLE and SPE methods. Our method offers a significant advantage due to the extensive surface area created by fine droplets of the extraction solvent in contact with urine samples caused by the use of dispersive solvent (DLLME) and the cycles of aspiration and dispersion through the use of a syringe (AALLME). Additionally, it requires lower solvent volumes for extraction compared to classical methods and offers a notably shorter running time than previously proposed techniques. Although the sample volume used in our current study was relatively higher than some other studies, we fully exploited the outstanding advantages offered by the proposed AADLLME method, specifically its simplicity. Furthermore, our method exhibited similar – if not higher – quality in terms of analytical performance, including sensitivity, extraction time, and chromatographic time analysis, compared to the other reported techniques.[9,29,31,32,47,52–54]

Table IV. Comparison of analytical performance between the developed method and previously reported methods for the determination of phthalate monoesters in urine samples

m-PAEs	Sample volume (mL)	Sample Preparation	Instrumental method	Solvent volume (µL)	LOD	LOQ	Running (min)	Ref.
15	2.5	AADLLME	LC-MS/MS	900 dichloroethane 1200 acetonitrile	0.01-0.05	0.03-0.17	11.0	This study
2	1.0	DLLME	LC-DAD	Methanol (350) $[C_6MIM][PF_6]$ (50)	0.96-3.20	3.1-10.6	30.0	49
6	5.0	DLLME	GC-MS	Acetonitrile (750) Chlorobenzene (80)	0.02-0.54	0.05-1.8	20.0	28
8	16	HF-LPME	GC-MS	Octanol (35)	0.78-23.3	1.29-38.9	45.2	31
8	0.5	SPME	GC-MS/MS	No solvent	-	0.5-5	31.0	30
25	0.5	SPE	LC-MS/MS	Acetonitrile (1200) Ethyl acetate (1100)	0.01-23.0	0.03-76.4	30.0	45
3	1.0	LLE	LC-MS/MS	Ethyl acetate (3000)	0.2-0.3	0.7-1.0	-	50
10	10	LLE	GC-MS	Toluene (4000)	0.1-0.4	0.3-1.3	35.0	51
19	1.0	Diluted-shoot	UHPLC-MS/MS	—	—	2.8-60	3.0	7

CONCLUSIONS

In this study, a fast and sensitive analytical method for determining 15 m-PAEs in urine samples was successfully developed and validated. The proposed method combines two appropriate sample pre-treatments, AALLME and DLLME, followed by LC-MS/MS determination. Under optimized conditions, determined by multivariate experimental designs, the method's performance showed good adjustments of the linear models by the ordinary least squares method, recoveries, repeatability, and intermediate precision. The proposed procedure is fast, simple, and highly sensitive, allowing the determination of m-PAEs at low concentrations. The good analytical characteristics, simplicity, and affordability of the procedure make it a suitable and attractive alternative method for routine studies of human exposure to phthalates.

Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this study.

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SUPPLEMENTARY MATERIAL

Table S1. Specific information regarding phthalate monoesters analyzed, their respective parent compound, chemical names, and their abbreviations

Major Parent phthalate	Phthalate metabolites	Abreviation	Supplier**
Dimethyl phthalate	Monomethyl phthalate	mMP	Sigma
Diethyl phthalate	Monoethyl phthalate	mEP	Sigma
Diisopropyl phthalate	Monoisopropyl phthalate	mIPrP	TRC
Di-n-propyl phthalate	Mono-n-propyl phthalate	mPrP	TRC
Di-butyl phtalate	Mono-butyl phthalate	mBuP*	CIL
Diisopentyl phthalate	Monoisopentyl phthalate	mIPeP	Campro
Dicyclohexyl phthalate	Monocyclohexyl phthalate	mCHP	Sigma
Di-n-octyl phthalate	Mono(3-carboxypropyl) phthalate	mCPP	TRC
Benzyl Butyl phthalate	Monobenzyl phthalate	mBzP	Sigma
Di(2-ethylhexyl) phthalate	Mono(2-ethylhexyl) phthalate	mEHP	CIL
Di(2-ethylhexyl) phthalate	Mono(2-ethyl-5-oxohexyl) phthalate	mEOHP	CIL
Di(2-ethylhexyl) phthalate	Mono(2-ethyl-5-hydroxyhexyl) phthalate	mEHHP	CIL
Di(2-ethylhexyl) phthalate	Mono[(2-carboxymethyl) hexyl] phthalate	mCMHP	TRC
Di(2-ethylhexyl) phthalate	Mono(2-ethyl-5-carboxypentyl) phthalate	mECPP	CIL
Diisononyl phthalate	Mono-carboxy-isooctyl phthalate	mCIOP	TRC

Phthalate metabolites	Internal standards	Abreviation	Supplier**
mMP	Monomethyl phthalate-*d*4	mMP-*d*4	TRC
mEP	Monoethyl phthalate-*d*4	mEP-*d*4	TRC
mIPrP	Mono-propyl phthalate-*d*4	mPrP-*d*4	TRC
mPrP	Mono-propyl phthalate-*d*4	mPrP-*d*4	TRC
mBuP*	Mono-n-butyl phthalate (ring-1,2-$^{13}C_2$, dicarboxyl-$^{13}C_2$)	mBuP-^{13}C	CIL
mIPeP	Mono-iso-pentyl phthalate-*d*4	mIPeP-*d*4	CDN
mCHP	Monocyclohexyl phthalate-*d*4	mCHP-*d*4	TRC
mCPP	Mono (3-carboxypropyl) phthalate-*d*4	mCPP-*d*4	TRC

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Table S1. Specific information regarding phthalate monoesters analyzed, their respective parent compound, chemical names, and their abbreviations (continuation)

Phthalate metabolites	Internal standards	Abreviation	Supplier**
mBzP	Monobenzyl phthalate-*d*4	mBzP-*d*4	TRC
mEHP	Mono-2-ethylhexyl phthalate (ring-1,2-$^{13}C_2$, dicarboxyl-$^{13}C_2$)	mEHP-^{13}C	CIL
mEOHP	Mono-(2-ethyl-5-oxohexyl)phthalate ($^{13}C_4$)	mEOHP-^{13}C	CIL
mEHHP	Mono-(2-ethyl-5-hydroxyhexyl)phthalate ($^{13}C_4$)	mEHHP-^{13}C	CIL
mCMHP	Mono [2- (carboxymethyl) hexyl] phthalate-*d*4	mCMHP-^{13}C	TRC
mECPP	Mono-(2-ethyl-5-carboxypentyl)phthalate ($^{13}C_4$)	mECPP-^{13}C	CIL
mCIOP	Mono-carboxy-isooctyl phthalate-*d*4	mCIOP-*d*4	TRC

*refers to the sum of metabolites of di-n-butyl phthalate and diisobutyl phthalate; **CIL: Cambridge Isotope Laboratories (Andover, MA, USA); TRC: Toronto Research Chemicals (Toronto ON, Canada); Sigma-Aldrich (St. Louis, MO, USA); Campro Scientific GmbH (Berlin, Germany); CDN Isotopes (Augsburg, Germany)

Table S2. LC-MS/MS acquisition parameters used for the analysis of the 15 phthalate monoesters

Phthalate metabolites	Precursor Ion	Quantif. Ion	Collision energy (eV)	Tube lens (V)	Retention time (min)**
mMP	178.969	77.432	20	54	4.42
mMP-*d*4	183.000	110.803	15	63	4.42
mEP	193.028	77.327	18	85	4.64
mEP-*d*4	197.028	81.327	18	85	4.64
mIPrP	207.010	77.228	20	73	4.96
mPrP	207.010	77.228	20	73	5.07
mPrP-*d*4	210.900	150.036	18	80	5.07
mBuP*	221.039	77.241	21	96	5.56
mBuP-^{13}C	225.000	137.415	18	74	5.57
mIPeP	235.000	191.137	14	80	5.94
mIPeP-*d*4	239.000	195.162	13	74	5.94
mCHP	247.061	97.199	19	72	6.00
mCHP-*d*4	251.088	97.211	18	97	6.00
mCPP	251.000	103.176	12	43	4.45
mCPP-*d*4	255.000	169.396	17	92	4.44

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Table S2. LC-MS/MS acquisition parameters used for the analysis of the 15 phthalate monoesters (continuation)

Phthalate metabolites	Precursor Ion	Quantif. Ion	Collision energy (eV)	Tube lens (V)	Retention time (min)**
mBzP	255.019	77.226	22	74	5.63
mBzP-*d*4	259.000	215.211	14	87	5.62
mEHP	277.050	134.124	18	100	7.14
mEHP-^{13}C	281.00	236.094	16	85	7.14
mEOHP	291.097	121.099	21	97	5.42
mEOHP-^{13}C	295.000	124.117	21	96	5.43
mEHHP	293.127	121.163	23	83	5.59
mEHHP-^{13}C	297.000	124.287	22	91	5.60
mCMHP	307.091	159.149	15	62	5.70
mCMHP-^{13}C	311.000	113.200	34	74	5.70
mECPP	307.091	159.149	15	62	5.40
mECPP-^{13}C	311.000	113.200	34	74	5.40
mCIOP	321.000	173.123	22	80	5.87
mCIOP-*d*4	325.026	173.381	18	54	5.86

*refers to the sum of metabolites of di-n-butyl phthalate and diisobutyl phthalate; min: minutes

Table S3. Central composite rotatable design based on four significant parameters and the extraction efficiency (geometric mean) of phthalate monoesters from synthetic urine sample as the response

Factor	Levels			Star point α=2	
	Low (-1)	Central (0)	High (+1)	-α	+α
X1: Extraction solvent volume (mL)	0.750	1.000	1.250	0.500	1.500
X2: Dispersive solvent Volume (mL)	0.500	1.000	1.500	0.000	2.000
X3: Number of extraction cycles	2	4	6	0	8
X4: Ionic strength (%w/v)	5	10	15	0	20

Factor					Extraction efficiency	
Run	X1	X2	X3	X4	Experimental	Predicted
1	0.75	0.5	2	5	42	44.3
2	1.25	0.5	2	5	53	53.2

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Table S3. Central composite rotatable design based on four significant parameters and the extraction efficiency (geometric mean) of phthalate monoesters from synthetic urine sample as the response (continuation)

	Factor				Extraction efficiency	
Run	**X1**	**X2**	**X3**	**X4**	**Experimental**	**Predicted**
3	0.75	1.5	2	5	70	68.0
4	1.25	1.5	2	5	69	67.2
5	0.75	0.5	6	5	46	43.7
6	1.25	0.5	6	5	54	54.8
7	0.75	1.5	6	5	69	71.2
8	1.25	1.5	6	5	68	72.5
9	0.75	0.5	2	15	53	48.7
10	1.25	0.5	2	15	54	54.8
11	0.75	1.5	2	15	64	66.2
12	1.25	1.5	2	15	60	62.5
13	0.75	0.5	6	15	52	56.8
14	1.25	0.5	6	15	63	65.2
15	0.75	1.5	6	15	78	78.0
16	1.25	1.5	6	15	76	76.7
17	0.5	1	4	10	64	64.1
18	1.5	1	4	10	75	71.6
19	1	0	4	10	35	34.3
20	1	2	4	10	72	69.5
21	1	1	0	10	52	53.6
22	1	1	8	10	72	67.1
23	1	1	4	0	62	61.6
24	1	1	4	20	73	70.1
25	1	1	4	10	82	81.7
26	1	1	4	10	84	81.7
27	1	1	4	10	79	81.7

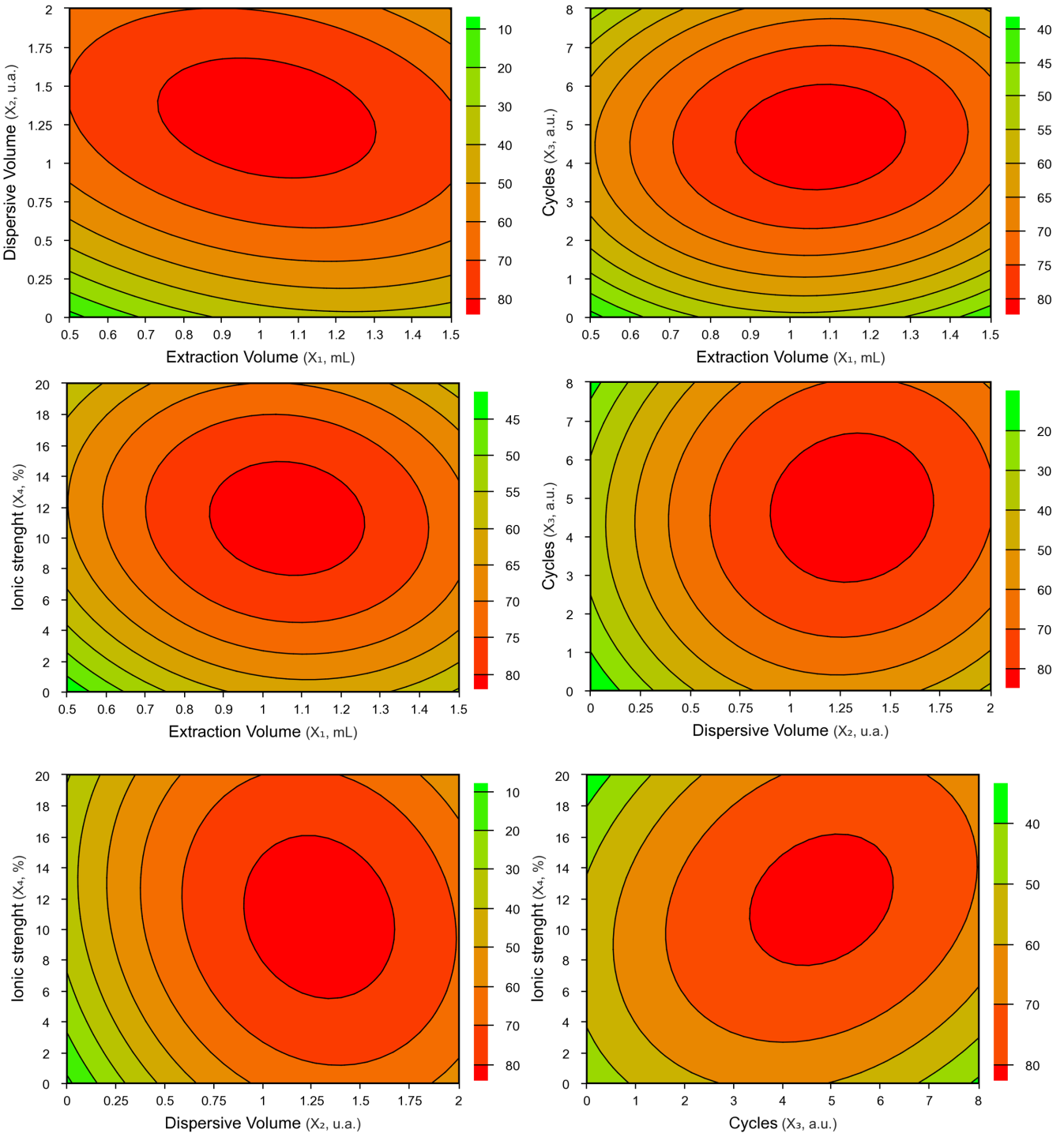

Figure S1. Contour plots of the extraction efficiency (%) of phthalate monoesters obtained by central composite rotatable design, as a function of extraction solvent volume, the number of extraction cycles and ionic strength.