



A simple and selective method for determination of phthalate biomarkers in vegetable samples by high pressure liquid chromatography–electrospray ionization–tandem mass spectrometry



Xi Zhou^a, Kunyan Cui^b, Feng Zeng^{a,*}, Shoucong Li^a, Zunxiang Zeng^a

^a School of Chemistry and Chemical Engineering, Sun Yat-sen University, Guangdong, Guangzhou 510275, China

^b Instrumental Analysis and Research Center, Sun Yat-sen University, Guangdong, Guangzhou 510275, China

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ABSTRACT

In the present study, solid-phase extraction cartridges including silica reversed-phase Isolute C₁₈, polymeric reversed-phase Oasis HLB and mixed-mode anion-exchange Oasis MAX, and liquid–liquid extractions with ethyl acetate, *n*-hexane, dichloromethane and its mixtures were compared for clean-up of phthalate monoesters from vegetable samples. Best recoveries and minimised matrix effects were achieved using ethyl acetate/*n*-hexane liquid–liquid extraction for these target compounds. A simple and selective method, based on sample preparation by ultrasonic extraction and liquid–liquid extraction clean-up, for the determination of phthalate monoesters in vegetable samples by liquid chromatography/electrospray ionisation–tandem mass spectrometry was developed. The method detection limits for phthalate monoesters ranged from 0.013 to 0.120 ng g^{−1}. Good linearity ($r^2 > 0.991$) between MQIs and 1000× MQIs was achieved. The intra- and inter-day relative standard deviation values were less than 11.8%. The method was successfully used to determine phthalate monoester metabolites in the vegetable samples.

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1. Introduction

Phthalates are a class of synthetic compounds mainly used as non-reactive plasticisers in polyvinylchloride (PVC), polyvinyl acetates, cellulose and polyurethanes, and as non-plasticisers in the manufacturing of cosmetics, paints, glues, photographic films, and insect repellents (Net, Sempéré, Delmont, Paluselli, & Ouddane, 2015; Staples, Peterson, Parkerton, & Adams, 1997). Due to widespread use of phthalate-containing products, phthalates have been detected in various environmental matrices (Adeogun et al., 2015; Hassanzadeh, Sari, Khodabandeh, & Bahramifar, 2014; Kim et al., 2015; Ma, Christie, Luo, & Teng, 2013; Meng et al., 2014; Morrison, Li, Mishra, & Buechlein, 2015; Wu et al., 2015; Zhang et al., 2015). Elevated levels of phthalates are ubiquitous in agricultural soils irrigated with treated wastewater, commonly called reclaimed or recycled water, and amended with biosolids (Kong et al., 2012; Niu, Xu, Xu, Yun, & Liu, 2014; Wang et al., 2015). Similar to other hydrophobic organic pollutants, phthalates may tend to accumulate in growing crops, potentially

causing damage to the crops, and/or entering the food chain (Liao, Yen, & Wang, 2009; Ma et al., 2013; Meng et al., 2014; Wang, Fan, & Wang, 2015; Wu et al., 2013; Yin, Lin, Wang, & Zhang, 2003). Currently, the occurrence, fate, and potential exposure risks of phthalates in vegetable/soil systems are causing increasing concern (Net et al., 2015; Wang et al., 2015).

Once in the vegetables, phthalates are rapidly metabolised by hydrolysis to their corresponding monoester metabolites. These phthalate monoester metabolites are non-persistent with short half-lives, which make them ideal biomarkers of the vegetables' exposure to phthalates (Högberg et al., 2008; Marie, Vendittelli, & Sauvant-Rochat, 2015; Net et al., 2015). In assessing vegetable exposure to phthalates, it is essential to have a sensitive and selective analytical method that can quantify phthalate monoester metabolites in vegetable samples. Recently, high pressure liquid chromatography–electrospray ionisation (ESI)–tandem mass spectrometry (LC–ESI–MS/MS) using multiple reaction monitoring (MRM) has emerged as an effective method for the analysis of phthalate monoester metabolites (Net et al., 2015). To date, LC–ESI–MS/MS has been applied to the determination of phthalate monoesters in urine, hair and milk samples (Chang, Lin, & Chang, 2013; Högberg et al., 2008; Kim et al., 2015; Servaes et al., 2013). However, to the best of our knowledge, there is no reported

* Corresponding author.

E-mail address: ceszf@mail.sysu.edu.cn (F. Zeng).

analytical method utilising LC–MS/MS for the determination of phthalate monoester metabolites in vegetable samples.

For the determination of phthalate monoester metabolites in vegetable samples by LC–ESI–MS/MS, sample preparation, including extraction and clean-up procedures, is required, in order to isolate phthalate monoester metabolites from the vegetable samples, remove interfering compounds and achieve a sufficient sensitivity. Nowadays, several extraction techniques have been tested for plant samples, including Soxhlet extraction (Rodríguez-Solana, Salgado, Domínguez, & Cortés-Diéguez, 2014), ultrasound-assisted extraction (UAE) (Rezaie, Farhoosh, Iranshahi, Sharif, & Golmohamadzadeh, 2015; Zhou et al., 2014), pressurized liquid extraction (PLE) (Shang, Kim, & Um, 2014), accelerated solvent extraction (ASE) (Rodríguez-Solana et al., 2014; Wang, Fan, et al., 2015) and microwave-assisted extraction (MAE) (Dahmoune, Nayak, Moussi, Remini, & Madani, 2015; Zhang et al., 2014). UAE is one of the upcoming extraction techniques that can offer high reproducibility in short times, simplified manipulation, reduced solvent consumption and temperature, and lower energy input (Rezaie et al., 2015). Ultrasonic cavitations create shear forces that disrupt cell walls mechanically and increase mass transfer processes. Moreover, there is no chemical reaction in UAE, which can prevent chemical degradation of target compounds (Zhou et al., 2014). However, the extraction efficiency of phthalate monoester metabolites in complex plant matrices depends on the extent to which they are associated with the pigments, lipids and other lipophilic matrix substances; the co-extraction of lipids and other lipophilic matrix substances can alter the ionisation efficiency of LC–ESI–MS/MS, which may lead to serious matrix effects (González-Curbelo, Herrera-Herrera, Ravelo-Pérez, & Hernández-Borges, 2012). Therefore, the clean-up of UAE extracts is a key procedure in the determination of phthalate monoester metabolites by LC–ESI–MS/MS. Solid-phase extraction (SPE) and liquid–liquid extraction (LLE) are the most common clean-up methods for phthalate monoester metabolites from biological samples (Chang et al., 2013; Itoh et al., 2009; Kim, Song, Choi, Lee, & Pyo, 2014; Monfort, Ventura, Balcells, & Segura, 2012). The objectives of this study were to (1) optimise, in terms of efficiency, the sample pre-treatment involved in UAE and SPE/LLE clean-up procedure, and (2) develop a simple and selective analytical method for the simultaneous determination of phthalate monoester metabolites in vegetable samples.

2. Experimental

2.1. Reagents and materials

Monomethyl phthalate (MMP), monoethyl phthalate (MEP), monoisobutyl phthalate (MiBP), mono-*n*-butyl phthalate (MnBP), monobenzyl phthalate (MBzP), mono-*n*-hexyl phthalate (MHP), mono-(2-ethylhexyl) phthalate (MEHP), mono-*n*-octyl phthalate (MnOP), *d*₄-mono-*n*-butyl phthalate (*d*₄-MnBP) and *d*₄-mono-2-ethylhexyl phthalate (*d*₄-MEHP) were purchased from CDN Isotopes, Inc. (Quebec, Canada). Isolute C₁₈ (6 cm³, 200 mg) SPE cartridges were obtained from Biotage (Uppsala, Sweden). Oasis HLB (6 cm³, 200 mg), and Oasis MAX (6 cm³, 200 mg) SPE cartridges were purchased from Waters (Milford, MA). HPLC-grade reagents, including acetone (Ac), *n*-hexane (Hex), acetonitrile (ACN), ethyl acetate (EtOAc), dichloromethane (DCM), methanol (MeOH), and formic acid (FA) were purchased from Sigma–Aldrich (St. Louis, MO). Milli-Q water, produced using a Milli-Q purification system (Millipore Corporation, Billerica, MA), extracted with DCM and Hex, respectively, and then redistilled. Laboratory glassware was soaked overnight in K₂CrO₇/H₂SO₄ solution, washed with tap water and redistilled water, baked at 300 °C for 12 h, and then rinsed with acetone, DCM and Hex, respectively.

2.2. Standard solution preparation

Individual stock standard solutions of the phthalate monoesters and *d*₄-MnBP were prepared at 0.1 mg mL^{−1} in MeOH or ACN. Mixed working solutions of the phthalate monoesters were prepared by dilution of the individual stock standard solutions in MeOH or ACN. All solutions were stored in darkness at −20 °C.

2.3. Biological material

Fresh cucumber (*Cucumis sativus* L.) was collected from suburban plastic film greenhouses in Guangzhou, China. The vegetable samples were weighed, cut into small pieces, homogenised, and then stored at −20 °C until analysis.

2.4. Sample extraction

The cucumber samples (10.00 g fresh weight (FW)) were weighed into 25-mL polytetrafluoroethylene centrifuge tubes, spiked with phthalate monoesters and *d*₄-MnBP, stored in dark at 4 °C overnight. Afterwards, the extraction solvents were added and samples placed in a 10 °C ultrasonic water bath (KH300SP, 25 kHz, 300 W; Kunshan Ultrasonic Instrument Co. Jiangsu, China) for 10 min. The organic phase was separated by centrifuging at 12,000g under 10 °C for 10 min. Three replicates were carried out. The combined extracts were evaporated to dryness. The following experiments were tested as clean-up protocols.

2.5. Sample purification

2.5.1. Liquid–liquid extraction

The extracted supernatants or the standard solutions were redissolved in 2.0 mL 0.50% (v/v) FA in water, extracted with 5.0 mL EtOAc, Hex, DCM and its solvent mixtures, respectively. The organic phases were collected. Three replicates were carried out. The combined extracts were evaporated to dryness, and reconstituted in 50 µL of mobile phase for LC–ESI–MS/MS analysis (Kim et al., 2014).

2.5.2. Oasis HLB cartridge

The extracted supernatants or the standard solutions were redissolved in 2.0 mL 0.5% (v/v) FA in water, loaded on Oasis HLB cartridges (6 cm³, 200 mg; Waters Corp., Milford, MA) preconditioned with 2.0 mL MeOH and 2.0 mL water. The cartridge was washed with 1.0 mL of water and 1.0 mL 40% MeOH in water, and phthalate monoesters were eluted with 2.0 mL of ACN. The eluates were dried and reconstituted in 50 µL of LC mobile phase for LC–ESI–MS/MS analysis (Chang et al., 2013).

2.5.3. Oasis MAX cartridge

The extracted supernatants or the standard solutions were redissolved in 2.0 mL 0.50% (v/v) FA in water, loaded on Oasis MAX cartridges (6 cm³, 200 mg; Waters Corp., Milford, MA) preconditioned with 2.0 mL ACN and 2.0 mL water. The cartridge was washed with 1.0 mL water and 2.0 mL ACN and phthalate monoesters were eluted using 2.0 mL 1.00% FA in ACN. The eluates were dried and reconstituted in 50 µL of LC mobile phase for LC–ESI–MS/MS analysis (Itoh et al., 2009).

2.5.4. Isolute C₁₈ cartridge

The extracted supernatants or the standard solutions were redissolved in 2.0 mL 1.0% FA in ACN, loaded on Isolute C₁₈ cartridges (6 cm³, 200 mg; Biotage, Sweden) preconditioned with 2.0 mL MeOH and 2.0 mL water. The cartridge was washed with 2.0 mL water, and phthalate monoesters were eluted using 2.0 mL 1.0% FA in ACN. The eluates were dried and reconstituted

in 50 μ L of LC mobile phase for LC–ESI–MS/MS analysis (Langer et al., 2014).

2.6. LC–MS/MS analysis

An Agilent 1260 series HPLC system (Agilent Technologies, Waldbronn, Germany) consisting of a degasser, binary pump, autosampler and column oven coupled to an Applied Biosystems–Sciex API 4500 (Applied Biosystems, Concord, Ontario, Canada) triple–quadrupole mass spectrometer was employed for the separation and quantification of phthalate monoesters.

Separation of phthalate monoesters was achieved on an Eclipse plus C₁₈ column (150 mm $\times$ 3.0 mm i.d., 3.5 μ m; Agilent Technologies) with a security guard pre–column (10 mm $\times$ 3.0 mm i.d., 3.5 μ m; Agilent Technologies). The column was equilibrated at 25 °C. The mobile phase is 0.001% FA in water (A)/MeOH (B) at a flow rate of 0.4 mL min^{–1}. The gradient profile started with 50% B for 4.0 min, linearly increased to 60.0% B in 0.10 min; held for 8.0 min, and then increased to 80.0% B in 0.10 min, held for 11.0 min before being returned to 50.0% B within 0.10 min, followed by a re–equilibration time of 7.0 min.

The MS parameters were optimised with mixtures of standard solutions. Nitrogen was used as the nebuliser, drying, and collision gases. The nebuliser gas pressure, drying gas pressure, curtain gas pressure, source voltage and source temperature were set at 50 psi, 60 psi, 40 psi, –5.0 kV and 600 °C, respectively. Quantification was performed in the MRM acquisition mode. Phthalate monoesters and their corresponding internal standards were analysed in negative ion mode.

Data were acquired and processed using Analyst 1.6.2 software (AB SCIEX, Foster City, CA).

2.7. Method validation

This method was validated by calculating the recovery, relative standard deviation (RSD), method detection limits (MDLs), and method quantification limits (MQLs) of phthalate monoesters in cucumber samples. The recoveries of phthalate monoesters in the cucumber samples were assessed by spiking a standard mixture at three concentrations (0.20, 2.00, and 10.00 ng g^{–1} FW). Precision was evaluated as the RSD of replicate measurements. Intra- and inter-day precisions were determined by assaying the crop materials spiked with high, intermediate and low levels of phthalate monoesters on five consecutive days with seven replicates each day. Standard calibration was performed by plotting analyte concentrations versus peak areas. MDLs and MQLs were determined at signal-to-noise ratios of 3 and 10, respectively.

2.8. Application to plant samples

The applicability of the established method was demonstrated by the determination of phthalate monoesters in the cucumber samples. The cucumber samples were collected from the suburban plastic film greenhouses in Guangzhou, China. All of the collected cucumber samples were weighed, freeze-dried, homogenised, and stored at –20 °C until sample extraction. Frozen cucumber samples were ground and then transferred into 25-mL centrifuge tubes. A solution of d₄–MnBP was added. Extractions, clean-ups, and analyses were performed using the established method.

3. Results and discussion

To ensure the precision and accuracy of the determination of phthalate monoesters in cucumber samples by LC–ESI–MS/MS

under MRM, the extraction and purification efficiencies were evaluated.

3.1. Evaluation and optimisation of ultrasonic extraction methods

Considering the physicochemical properties of phthalate monoester metabolites and previous reports (Chang et al., 2013; Högberg et al., 2008; Kim et al., 2015; Net et al., 2015; Tranfo et al., 2012), the polar organic solvents MeOH, ACN, Ac and their water mixtures, were selected as the ultrasonic extraction solvents of phthalate monoesters in the spiked cucumber samples. Non-spiked vegetable materials were also processed in parallel as sample blanks. Ultrasonic extraction was performed in an ultrasonic bath at 10 °C for 10.0 min. Three replicates were carried out.

For MeOH, ACN and Ac, the ultrasonic extract chromatograms presented many interfering peaks but only small amounts of phthalate monoesters were obtained from the spiked cucumber samples. No improvement of the extraction efficiencies of the phthalate monoesters was obtained for the organic solvent mixtures, such as MeOH/Ac (1:1, v/v), ACN/Ac (1:1, v/v) and MeOH/ACN (1:1, v/v), than for individual solvents. Amongst these test solvent mixtures, MeOH/H₂O mixtures displayed better recoveries for phthalate monoesters ($p < 0.05$), with the least amount of interfering substances, including pigments, lipids, and other lipophilic matrix substances. Most efficient extractions of phthalate monoesters were obtained with more than 50.0% MeOH in the MeOH/H₂O mixtures (Table 1). A comparison between acidified and non-acidified MeOH/H₂O mixtures (7:3, v/v) revealed that slightly higher recoveries of phthalate monoesters were achieved by MeOH/H₂O mixtures (7:3, v/v) acidified with 1.0% FA.

Combinations of different solvent volumes of the 1.0% FA in MeOH/H₂O mixtures (7:3, v/v, 5.0–40.0 mL) and number of extraction cycles (up to six) were extensively tested. Finally, good recovery of phthalate monoesters was achieved using 10 mL the 1.0% FA in MeOH/H₂O mixtures (7:3, v/v) and three extraction cycles.

3.2. Evaluation and optimisation of purification methods

Common solid-phase extraction cartridges, including polymer-based reversed-phase sorbents (e.g., Oasis HLB cartridges: Kim et al., 2015; Servaes et al., 2013), silica-based reversed-phase sorbents (e.g., Isolute C₁₈ cartridges: Dirtu et al., 2013; Langer et al., 2014; Tranfo et al., 2012) and polymer-based mixed-mode anion-exchange sorbent (e.g., Oasis MAX cartridges: Callesen et al., 2014; Itoh et al., 2009), and EtOAc, Hex, and DCM LLEs (Chang et al., 2013; Kim et al., 2014; Monfort et al., 2012) were used in this paper to clean up MeOH/H₂O extracts of phthalate monoesters for their determination by LC–ESI–MS/MS. The studied SPEs and LLEs were tested using working standard solutions containing labelled and unlabelled phthalate monoesters, and recoveries varied from 76.7% to 103.9%.

Under optimised conditions, the clean-up efficiencies of the studied SPEs and LLEs were evaluated by analysing the acidified MeOH/H₂O extracts of cucumber samples spiked with phthalate monoesters at two concentration levels (0.10 and 1.00 ng g^{–1}). As shown in Table 1, the clean-up efficiencies obtained by the SPEs were unsatisfactory, especially the low extraction efficiencies obtained for MMP, MEP, MiBP and MnBP, whose recoveries ranged from 37.6% to 51.9%.

For the LLEs, Hex LLEs resulted in recoveries ranging from 50.9% (MMP) to 80.5% (MEHP). DCM LLEs produced slightly improved recoveries compared to Hex LLEs. In contrast, when EtOAc was used as an extracting solvent, relatively polar phthalate monoesters such as MEP, MMP were effectively extracted (>65%), yet non-analyte polar substances such as pigments, lipids, and other lipophilic matrix substances were also excessively extracted,

Table 1Evaluation of the sample pretreatment methods for the determination of phthalate monoesters in cucumber (*Cucumis sativus* L.) samples by LC–ESI–MS/MS.

PME ^a	Recovery/% (mean, n = 3)																																	
	Sample extraction														Sample purification																			
															SPE ^b			LLE ^c																
	Ac ^d		Ac/H ₂ O			ACN ^e			ACN/H ₂ O			MeOH ^f			MeOH/H ₂ O			HyperSep C ₁₈	Oasis HLB	Oasis MAXs	EtOAc ^g	DCM ^h	Hex ⁱ	EtOAc/Hex					DCM/Hex					
	(80/ 20, v/v)	(60/ 40, v/v)	(50/ 50, v/v)	(40/ 60, v/v)	(20/ 80, v/v)	(80/ 20, v/v)	(60/ 40, v/v)	(50/ 50, v/v)	(40/ 60, v/v)	(20/ 80, v/v)	(80/ 20, v/v)	(60/ 40, v/v)	(50/ 50, v/v)	(40/ 60, v/v)	(20/ 80, v/v)	(90/ 10, v/v)	(70/ 30, v/v)							(50/ 50, v/v)	(30/ 70, v/v)	(10/ 90, v/v)	(90/ 10, v/v)	(70/ 30, v/v)	(50/ 50, v/v)	(30/ 70, v/v)	(10/ 90, v/v)			
MMP	18.4	20.7	24.3	22.4	18.1	12.4	20.8	21.4	23.4	18.8	12.8	12.1	30.7	40.9	41.7	40.5	32.7	12.9	42.4	43.7	37.6	65.5	61.1	50.9	75.9	89.3	83.6	62.7	52.6	70.2	68.3	58.4	31.8	25.3
MEP	19.3	20.1	25.4	23.4	16.2	9.68	19.7	20.9	21.1	20.7	19.5	20.2	32.5	38.4	35.9	36.7	29.8	22.7	51.9	48.2	42.9	69.5	66.4	54.7	79.6	91.9	87.1	64.9	64.8	72.9	70.2	61.6	42.5	30.9
MnBP	22.6	22.2	20.9	19.6	13.7	10.2	31.9	30.1	24.6	22.9	13.4	12.8	38.4	42.8	44.6	40.8	31.4	22.6	67.3	58.1	56.4	101.7	87.6	62.5	97.3	102	90.8	79.6	65.9	88.3	82.9	71.4	52.6	47.3
MiBP	35.8	35.4	31.8	26.3	14.1	10.9	39.7	35.2	35.1	21.3	18.7	20.8	42.2	44.9	51.2	46.3	38.7	28.7	71.9	66.1	45.6	95.9	87.4	69.7	100.4	94.8	87.9	80.4	71.6	82.9	85.1	72.5	61.3	56.2
MBzP	30.1	29.3	26.7	25.1	12.9	11.2	36.5	36.1	35.7	29.8	13.1	12.7	40.1	39.8	42.6	40.7	32.9	19.8	58.7	72.4	61.3	89.3	95.1	72.4	90.7	92.4	90.3	86.5	75.1	90.6	91.6	89.1	75.8	70.1
MHP	33.4	30.1	29.5	28.4	20.1	15.3	38.3	32.9	31.9	21.4	14.5	13.4	43.7	47.2	53.2	51.5	41.2	20.4	63.2	68.4	56.9	93.9	89.7	67.1	85.1	95.7	103	89.7	78.4	91.9	92.4	91.4	80.7	82.7
MEHP	32.9	31.6	30.8	25.4	20.4	14.9	37.6	36.7	42.9	19.8	13.9	12.8	39.4	45.1	43.9	41.3	30.5	19.6	79.3	53.7	54.2	92.5	76.1	80.5	98.6	113	90.2	80.6	79.1	73.7	88.9	75.7	75.9	72.8
MnOP	30.4	31.2	29.7	26.1	16.3	12.4	32.9	30.6	41.7	20.1	12.7	11.7	40.5	51.3	46.1	43.2	29.8	20.1	81.6	74.3	75.0	101.3	76.4	73.1	102.1	92.8	87.5	72.1	71.6	66.9	84.6	88.7	80.2	72.6
d ₄ -MnBP	35.2	34.3	32.6	30.8	12.8	10.7	37.6	36.3	32.9	18.4	19.4	18.5	42.7	50.1	51.9	48.4	40.7	21.8	72.9	69.7	70.2	95.7	85.6	62.6	95.1	101	89.3	81.3	80.3	71.4	75.1	75.9	75.3	72.7
d ₄ -MEHP	32.8	30.9	29.7	22.1	11.9	11.8	34.7	32.8	31.3	16.9	12.1	14.1	41.8	52.2	50.3	44.9	32.8	18.5	78.1	80.4	78.0	93.6	75.6	72.9	98.3	94.6	89.9	88.4	85.5	70.9	74.6	61.6	74.5	71.9

^a Phthalate monoesters: monomethyl phthalate (MMP), monoethyl phthalate (MEP), monoisobutyl phthalate (MiBP), mono-*n*-butyl phthalate (MnBP), monobenzyl phthalate (MBzP), mono-*n*-hexyl phthalate (MHP), mono-(2-ethylhexyl) phthalate (MEHP), mono-*n*-octyl phthalate (MnOP), d₄-mono-*n*-butyl phthalate (d₄-MnBP), d₄-mono-(2-ethylhexyl) phthalate (d₄-MEHP).

^b Solid-phase extraction.

^c Liquid–liquid extraction.

^d Acetone.

^e Acetonitrile.

^f Methanol.

^g Ethyl acetate.

^h Dichloromethane.

ⁱ Hexane.

Table 2Analytical validation of UAE-LLE-LC-ESI-MS/MS for the determination of phthalate monoesters in cucumber (*Cucumis sativus* L.) samples.

PME ^a	0.20 ng g ⁻¹ (FW) ^b			1.00 ng g ⁻¹ (FW)			10.0 ng g ⁻¹ (FW)			MDL ^c (ng/g)	MQL ^d (ng/g)
	Recovery (%)	Intra-day precision (RSD ^e , %; n = 7)	Inter-day precision (RSD, %; n = 5)	Recovery (%)	Intra-day precision (RSD, %; n = 7)	Inter-day precision (RSD, %; n = 5)	Recovery (%)	Intra-day precision (RSD, %; n = 7)	Inter-day precision (RSD, %; n = 5)		
MMP	115	7.02	2.97	82.9	5.01	6.04	78.3	10.7	8.17	0.092	0.304
MEP	80.9	3.42	8.41	89.2	10.4	13.2	81.0	5.21	4.21	0.120	0.396
MnBP	93.6	2.41	7.56	98.4	2.31	2.35	96.1	3.23	6.48	0.040	0.132
MiBP	90.0	4.72	5.29	96.5	5.11	6.33	99.7	5.27	5.73	0.032	0.106
MBzP	83.3	7.02	3.78	90.5	3.01	4.11	85.5	4.19	4.90	0.056	0.185
MHP	78.7	6.21	10.1	91.5	6.19	10.9	84.7	5.08	5.62	0.018	0.059
MEHP	88.8	3.91	8.98	98.2	4.85	7.14	86.1	3.77	4.38	0.048	0.158
MnOP	75.7	3.02	2.87	94.5	3.79	2.55	104	4.29	5.83	0.024	0.079
d ₄ -MnBP	87.2	5.23	9.48	97.3	5.18	8.21	83.9	7.74	6.16	0.017	0.057
d ₄ -MEHP	96.1	6.79	6.52	102	3.22	3.99	99.4	5.13	11.8	0.013	0.043

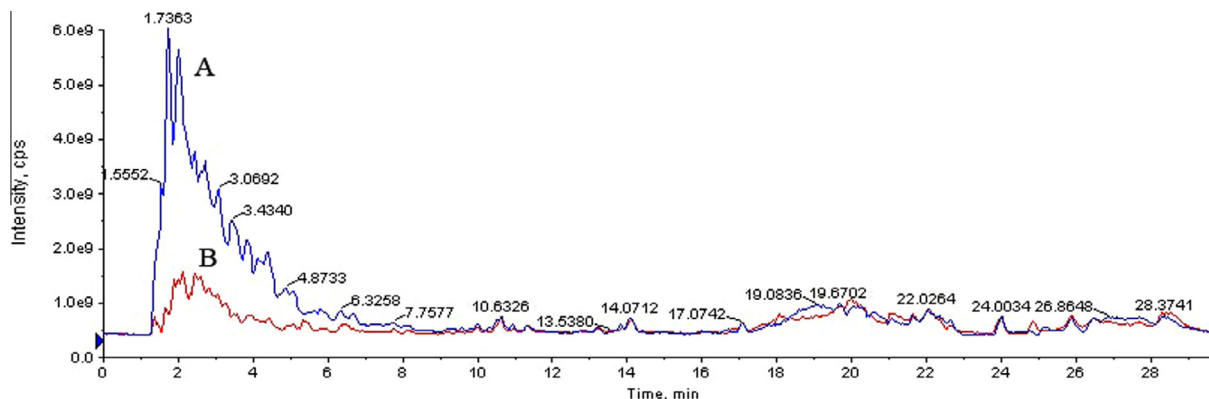
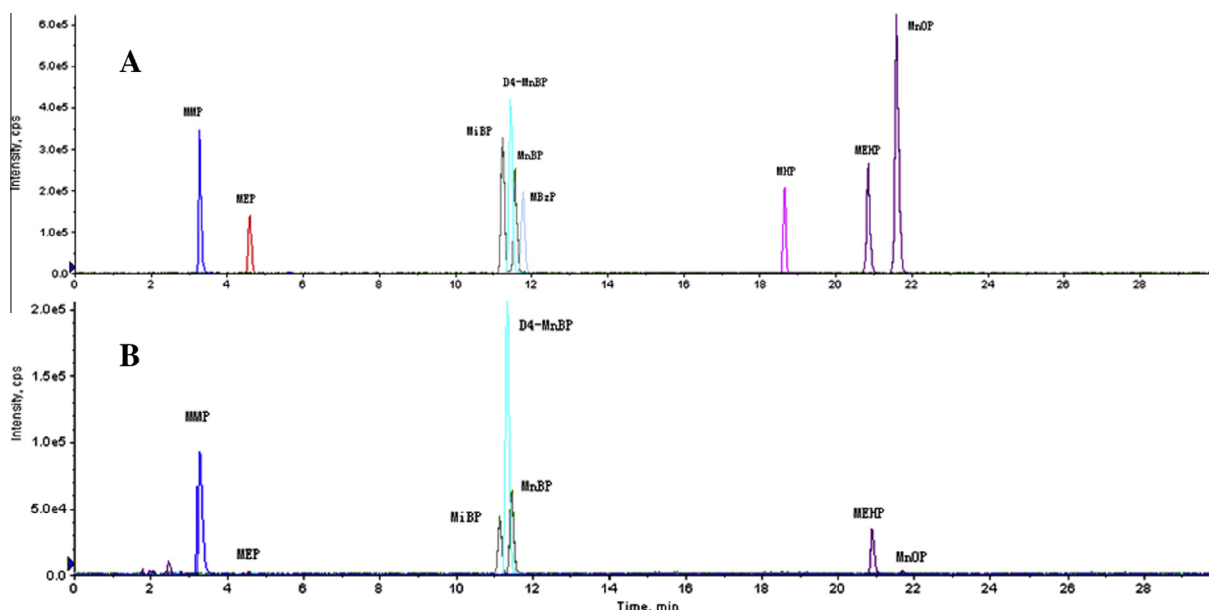
^a Phthalate monoesters: monomethyl phthalate (MMP), monoethyl phthalate (MEP), monoisobutyl phthalate (MiBP), mono-*n*-butyl phthalate (MnBP), monobenzyl phthalate (MBzP), mono-*n*-hexyl phthalate (MHP), mono-(2-ethylhexyl) phthalate (MEHP), mono-*n*-octyl phthalate (MnOP), d₄-mono-*n*-butyl phthalate (d₄-MnBP), d₄-mono-(2-ethylhexyl) phthalate (d₄-MEHP).

^b Fresh weight.

^c Method detection limit.

^d Method quantification limit.

^e Relative standard deviation.

**Fig. 1.** Total ion chromatograms of the methanol extracts of cucumber (*Cucumis sativus* L.) samples purified with Isolute C₁₈ SPE (A) and EtOAc/Hex LLE (70/30, v/v) (B).**Fig. 2.** MRM chromatogram of phthalate monoesters in a 5.00 ng mL⁻¹ standard solution (A) and in a cucumber (*Cucumis sativus* L.) sample (B) by LC-MS/MS.

which could cause matrix effects and affect the accuracy of phthalate monoester metabolites determination by LC–MS/MS. EtOAc and Hex were mixed (7:3, v/v) and used as extracting solvents; all phthalate monoesters were effectively extracted, with recoveries ranged from 89.3% (MMP) to 113% (MEHP), and interfering effects were small. Our results also demonstrated that less chromatographic background noise was achieved using ethyl acetate/*n*-hexane (7:3, v/v) (Fig. 1), resulting in increased S/N ratios for several target compounds.

In this study, 5.0 mL EtOAc/Hex (7:3, v/v) with two extraction cycles was finally selected as the optimal LLE procedure for the purification of phthalate monoesters in MeOH/H₂O extracts of cucumber samples. To determine lower levels of phthalate monoesters accurately in vegetable samples, it is very important to reduce matrix interference where possible. EtOAc/Hex LLE technique, as a routine sample preparation method, proved to be very efficient and useful for the extraction and clean-up of phthalate monoester metabolites in vegetable samples.

3.3. Method validation

To validate the analytical methodology, the linearity, accuracy, precision, MDLs, and MQLs of the phthalate monoesters were investigated (Table 2).

Method accuracy was expressed as the recovery of the phthalate monoesters and the internal standards, which ranged from 75.7% to 115%, 82.9% to 102% and 78.3% to 104% for the cucumber samples at spiking concentrations of 0.20, 1.00 and 10.0 ng g⁻¹ (FW). The intra- and inter-day precisions ranged from 2.31% to 10.7% and 2.35% to 11.8%, respectively. The MDLs obtained for the phthalate monoesters in the cucumber samples ranged from 0.013 to 0.120 ng g⁻¹. All of the phthalate monoesters were linear, with the squared regression coefficients (*r*²) greater than 0.991, between the MQLs and 1000× MQLs. These results demonstrate the suitability of the developed method for the analysis of phthalate monoesters in vegetable samples.

3.4. Application

The method developed in this work was applied to the determination of phthalate monoesters, including MMP, MEP, MnBP, MiBP, MBzP, MHP, MEHP and MnOP, in cucumber samples collected from the suburban plastic film greenhouses in Guangzhou, China. The surrogate standard used was d₄-MnBP and the cucumber samples were analysed in triplicate. A chromatogram of a cucumber sample is shown in Fig. 2(B). The surrogate standard recoveries of d₄-MnBP were 86.1 ± 8.22% in the vegetable samples. Among the collected vegetable samples, the detected concentrations ranged from 1.77 to 7.35 ng g⁻¹ FW for MMP, 0.22 to 0.86 ng g⁻¹ FW for MEP, 2.99 to 9.66 ng g⁻¹ FW for MnBP, 1.62 to 4.77 ng g⁻¹ FW for MiBP, MDL to 5.53 ng g⁻¹ FW for MEHP and MDL to 0.32 ng g⁻¹ FW for MnOP. MBzP and MHP were not detected. The concentrations of the phthalate monoesters were not surrogate-recovery corrected.

4. Conclusions

In this work, a new method for the simultaneous determination of phthalate monoester metabolites in cucumber (*C. sativus* L.) samples using HPLC–ESI–MS/MS was developed. Widely-used extraction and clean-up procedures were optimised and compared with respect to the extraction efficiency of phthalate monoesters. UAE coupled with EtOAc/Hex LLE clean-up procedures could effectively eliminate the lipids and other lipophilic matrix substances in complex biological matrices, which is important for the analysis of phthalate monoesters by LC–ESI–MS/MS. The developed method has been successfully applied to the determination of phthalate

monoester metabolites (MMP, MEP, MnBP, MiBP, MBzP, MHP, MEHP and MnOP) in cucumber samples, demonstrating the applicability of this method for the analysis of phthalate monoester metabolites in vegetables.

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