Simultaneous Determination of Nine Types of Phthalate Residues in Commercial Milk Products Using HPLC–ESI-MS–MS

Zhuokun Li[1], Feng Xue[2], Liguang Xu[1], Chifang Peng[1], Hua Kuang[1,*], Tao Ding[2], Chuanlai Xu[1], Chongyu Sheng[2], Yuxia Gong[2], and **Libing Wang[1]**

[1]School of Food Science and Technology, Jiangnan University, Wuxi, 214122, China, and [2]Jiangsu Entry-Exit Inspection and Quarantin Bureau, Nanjing, 210001, China

Abstract

A multi-residue method was developed for the confirmation and quantitation of nine types of phthalates in milk using high-performance liquid chromatography electrospray ionization tandem mass spectrometry. The samples were extracted with acetonitrile. The analytes were separated using a 0.1% formic acid-methanol system as the mobile phase, and a linear gradient elution program. Mass spectral acquisition was achieved by selectively monitoring the ions in electro-spray ionization mode. Qualitative analysis was based on the retention time and the mass spectrum results, and the quantity was carried out by comparison with the external standard. The mean recoveries for each analyte ranged from 65.2% to 98.3%, with relative standard deviations below 11.2%. The limits of detection were 5~25 μg/kg, and the limits of quantitation were 17~83 μg/kg, depending on the compounds. This method has the merits of convenient operation, high sensitivity, and good repeatability, making it an effective method for analysis of phthalates in milk. And the proposed analytical method has been applied to the analysis of phthalates presented in four commercial milk products. The main phthalate residues were DBP and DMP. And the amount of DBP was found to be more than 100 μg/kg in all this milk products.

Introduction

Phthalic acid diesters (PAEs), commonly referred to as phthalates, are a group of chemical compounds widely used in cosmetic products, food packaging materials, and polyvinyl chloride plastics. They are ubiquitously used as plasticizers to improve flexibility, pliability and elasticity in a broad range of plastic products. Phthalates are not chemically bound in the plastics, so they can be released into the environment. The general population may be exposed to them via ingestion, inhalation, or dermal routes (1).

Phthalates have long been suspected of being potentially disruptive to the endocrine system (2). Toxicological studies in animal models have demonstrated that some phthalates are developmental and reproductive toxicants (3,4). Yet limited human data are available on the possible relationship between phthalates and adverse reproductive effects (5–8). Due to their potential hazards and wide usage, the European Union has imposed an emergency restriction on the use of 6 phthalates (DBP, BBP, DEHP, DOP, DINP, and DIDP) in the production of items intended for children in the age group of 0–3 years (9).

Recently there has been increasing interest in monitoring phthalate compounds. Analytical methods have been published for determination of phthalates in a very broad range of matrices (10–12). The analysis of phthalic acid diesters is mostly carried out by high-performance liquid chromatography (HPLC) with diode array detection (13, 14), gas chromatography mass spectrometry (GC–MS) (15–18), LC–MS (19,20), LC–MS–MS (21,22), and other analyses.

Phthalate contamination, a major problem in phthalate analysis, which possibly results in over-estimation of the concentrations, should be taken into account. It may result in false positive results or over-estimation of the concentrations. The risk of contamination is present during the whole analytical procedure, including sample preparation and chromatographic analysis. Generally, glassware and organic solvents are the most important sources of contamination. Glassware and solvents can even be contaminated by laboratory air because volatile phthalates, particularly DEP, DiBP, and DBP, may cause severe environmental contamination. Commercially available organic solvents also contain trace amount of phthalates. On the other hand, in-house-distilled solvents are not always available for routine analysis and the risk of contamination is still present during and after solvent distillation. The major sources of contamination in the chromatographic system are located in the inlet and gas supply systems (1,23–29).

Various sample extraction methods for phthalates have been studied. Solid-phase extraction (SPE) methods have been reported for phthalates in urine by previously reports (30–36). However, most commercial cartridges have polyethylene or polypropylene barrels, leading to relatively high background levels of phthalates, especially DIOP. Glass barrels are available for packing with ODS material but it is not always possible to use these in routine analysis. Furthermore, SPE methods are relatively time-consuming, and require a certain volume of solvent for the elution step, thus exacerbating the problem of contamination. In addition, the lipophilic properties of most phthalates make them more complex.

*Author to whom correspondence should be addressed: email khecho@163.com.

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Generally, the GC–MS method needs complex derivatization steps and it may introduce more contamination to the analysis, and the LC–MS approach demands simpler clean-up procedures and shorter analysis times, and provides superior selectivity for the isomeric mixtures. In our research the milk samples were used as objects and the highly sensitive HPLC–ESI-MS–MS method for the simultaneous determination of 9 phthalates residues was developed.

Experimental

Chemicals

Standards of the individual phthalate esters: dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP), di-n-pentyl phthalate (DPP), dicyclohexyl phthalate (DCHP), di-*n*-octyl phthalate (DOP), diisodecyl phthalate (DIDP), diisooctyl phthalate (DIOP), and butyl benzyl phthalate (BBP) were purchased from Sigma-Aldrich (St. Louis, MO). Lichrosolv gradient grade acetonitrile and methanol were supplied by Merck (Darmstadt, Germany). Double-distilled water was produced by twice distilling ultrapure water; the ultrapure water was first purified using a Milli-Q Plus apparatus (Millipore, Bedford, MA). Ammonium acetate and formic acid were purchased from Sinopharm Chemical Reagent Co. (Shanghai, China). All reagents were analytical grade. All reagents and water were checked for contamination with phthalates before use.

Instrumentation

Phthalate separation and quantitation were carried out using a Thermo Scientific Surveyor Plus HPLC–MS–MS system (Waltham, MA). The platform comprised of a Thermo Scientific Surveyor HPLC Pump Plus, a Thermo Scientific Surveyor Autosampler Plus and a TSQ Quantum Access triple quadrupole mass spectrometer with electrospray ionization (ESI). A BP211D electronic balance was purchased from Sartorius Scientific Instruments Co., Ltd. (Göttingen, Germany). The nitrogen evaporator with a heating bath was obtained from Organomation Associates, Inc (Berlin, MA).

Chromatographic conditions and mass spectrometry

The separation of phthalates was carried out using a Finnigan Surveyor system and a Hypersil Gold Column (1.9 µm, 2.1 × 50 mm, Thermo, Palo Alto, CA). The column temperature was maintained at 25°C. The gradient mobile phase consisted of water, ammonium acetate, formic acid and methanol. The flow rate was 0.25 mL/min, and the injection volume was 25 µL. The specific conditions are shown in Table I. MS analysis was carried out using a TSQ Quantum Access mass spectrometer (Thermo Scientific, Waltham, MA) in select reaction monitoring (SRM) mode. The interface was operated in positive ion mode at a spray voltage of 4.5 kV and a capillary temperature of 350°C. The auxillary gas and sheath gas flow rates were set at 5 L/h and 20 L/h, respectively. ESI settings were optimized for each compound, as shown in Table II.

Table I. Linear Gradient Mobile Phase for LC

Time (min)	Methanol	5 mmol Ammonium acetate solution + 0.1% v/v formic acid
0–2	20%	80%
2–3.5	20–95%	80–5%
3.5–11	95%	5%
11–12	95–20%	5–80%
12–13.5	20%	80%

Table II. ESI Settings for HPLC–MS–MS to Detect Phthalates

English Name	Structure	Molecular weight	Parent ion (*m/z*)	Daughter ions (*m/z*)
Dimethyl phthalate (DMP)		$C_{10}H_{10}O_4$ = 194.1	195	**163**, 77 135,133
Diethyl phthalate (DEP)		$C_{12}H_{14}O_4$ = 222.4	223	**149**, 121 93, 65
Dibutyl phthalate (DBP)		$C_{16}H_{22}O_4$ = 278.3	279	**149**, 93 121, 65
Di-N-pentyl phthalate (DPP)		$C_{18}H_{26}O_4$ = 306.4	307	**149**, 121 93
Dicyclohexyl phthalate (DCHP)		$C_{20}H_{26}O_4$ = 330.4	331	**149**, 121 93, 65
Di-n-octyl phthalate (DOP)		$C_{24}H_{38}O_4$ = 390.5	413	**301**, 189 317
Diisodecyl phthalate (DIDP)		$C_{28}H_{46}O_4$ = 446.6	447	**149**, 415 121, 335
Diisooctyl phthalate (DIOP)		$C_{24}H_{38}O_4$ = 390.5	391	**149**, 121 93
Butyl benzyl phthalate (BBP)		$C_{19}H_{20}O_4$ = 312.3	313	**149**, 91 65

* Fragment ions for quantitation are bold.

Standard and working solution

Individual standard solutions of each phthalate at a concentration of 1 mg/mL were prepared in methanol in a Teflon-capped glass vial. From these solutions, a working mixture in methanol was prepared containing all the standards at a concentration of 10 µg/mL each. All the working solutions were freshly prepared on a daily basis by diluting this stock solution. The stock and standard solutions were preserved in the dark at 4°C. Calibration standards containing 10, 20, 50, 100, 200, and 400 ng/mL of the phthalates in methanol were prepared daily.

Sample preparation

All sample containers, measuring cylinders, centrifuge tubes, pipettes, etc., which came into contact with sample materials, sample extracts, standards, solvents and reagents, were made of glass. In order to reduce background contamination, all glassware was thoroughly washed with hot water, rinsed with ultrapure water, methanol, hexane and acetonitrile, respectively. The glassware was then baked at 400°C for at least 10 h. After cooling, the glassware was sealed with clean aluminum foil to avoid adsorption of phthalates from the air. Prior to use, the glassware was rinsed with a small aliquot of acetonitrile to deactivate the surfaces because the thermal treatment at 400°C resulted in the glassware surfaces adsorbing phthalates more readily.

For the determination of phthalate in whole milk samples (which were purchased from supermarket and packaged in plastic), the following sample preparation method was developed. An aliquot (2 g) of milk was transferred to a 50 mL centrifuge tube and 10 mL acetonitrile was then added to the samples. The mixture was homogenised in a vortex agitator. Sodium chloride (0.8 g) was then added to the solution which was vortexed once more. After shaking for 15 min and sonication for 10 min, the mixture was centrifuged at 1694 g for 10 min. The supernatant was decanted into another 50 mL centrifuge tube and evaporated under a vacuum rotary evaporator to dryness. The dry residue was re-dissolved in methanol to a final volume of 1.0 mL and frozen to remove sodium chloride precipitation before injection. The extract could not be filtered. The recovery experiments were carried out by adding different concentrations of phthalate standard solutions to real milk samples. The blank control sample of raw milk was prepared by the same sample preparation method, but didn't add phthalate standard solutions to the sample. The limit of detection (LOD) was calculated on the basis of the signal-to-noise ratio (S/N) of 3:1. The quantitation of phthalates was achieved using external standard calibration. The limit of quantitation (LOQ) was determined by adding the standard solution to a raw milk sample.

Results

Linearity, LOD, and LOQ

A standard solution series ranging in concentration from 10 to 400 ng/mL was prepared by diluting the stock solutions in methanol. The standard solutions were then directly injected in triplicate. Peak areas were plotted versus the amount of the analyte injected, and linear regression equations were applied. The linear ranges were ~ 0.010–0.400 µg/mL, and the range of correlation coefficients varied from 0.9913 to 0.9998, as showing in Table III. The results obtained for LODs and LOQs are demonstrated in Table III. As can be seen in Table III, LODs were between 5–25 ng/mL for all compounds, and the highest levels obtained were for DBP, DMP and DIOP. Total ion chromatograms obtained from a spiked milk sample (100 µg/kg) and a method blank are shown in Figures 1 and 2, respectively. The peaks demonstrated sharp and symmetrical peak shapes.

Recovery and accuracy

The recovery rate of the proposed method was evaluated by spiking raw milk samples with different concentrations (100, 200, 300, and 400 ng/mL) of phthalate standard solutions. The sample preparation process was carried out using the previously-mentioned pretreatment method. The reconstituted samples were analyzed and the results shown in Table III demonstrated that the average recoveries of phthalates ranged from 65.28% to 98.33%. The reproducibility of the analytical method was good and the relative standard deviation values were less than 11.22%.

Analytical applications

The proposed analytical method has been applied to the analysis of four commercial brands of milk products in order to detect these phthalates, and to verify the applicability and reliability of this method with real samples. The sample preparation process is described previously. The results obtained for these phthalates in the four milk samples tested are given in Table IV. The amount of DBP residue was found to be more than 100 µg/kg in all this milk products. In addition, the commercial milk samples showed common existence of DMP, but DIDP was not detected.

Table III. Linear Range, Correlation Coefficients, Average Recoveries, RSD, LODs, and LOQs Values Obtained from the Standard Addition Method in Milk Products

Phthalate	Linear range (µg/mL)	Correlation coefficient (R^2)	Average Recoveries	RSD	LOD (µg/kg)	LOQ (µg/kg)
DMP	0.030–0.400	0.9986	98.3%	5.3%	15	50
DEP	0.010–0.400	0.9990	68.7%	6.6%	7	23
DBP	0.050–0.400	0.9996	80.5%	8.5%	25	83
DPP	0.010–0.400	0.9998	67.2%	10.1%	5	17
BBP	0.010-0.400	0.9997	73.1%	9.7%	5	17
DCHP	0.010–0.400	0.9995	66.7%	8.7%	5	17
DIOP	0.020–0.400	0.9982	81.6%	11.2%	20	67
DOP	0.020–0.400	0.9913	65.8%	8.2%	15	50
DIDP	0.010–0.400	0.9989	65.2%	7.9%	9	30

Discussion

Liquid–liquid extraction was applied to sample preparation in our approach and the method was

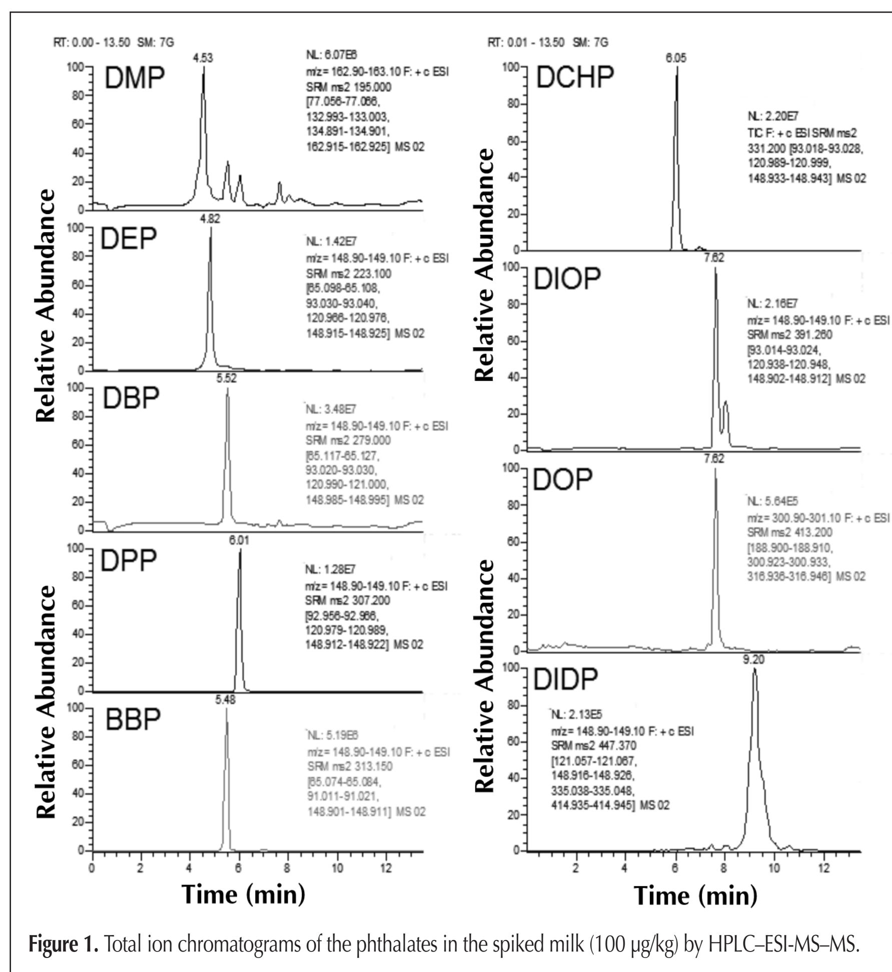

Figure 1. Total ion chromatograms of the phthalates in the spiked milk (100 μg/kg) by HPLC–ESI-MS–MS.

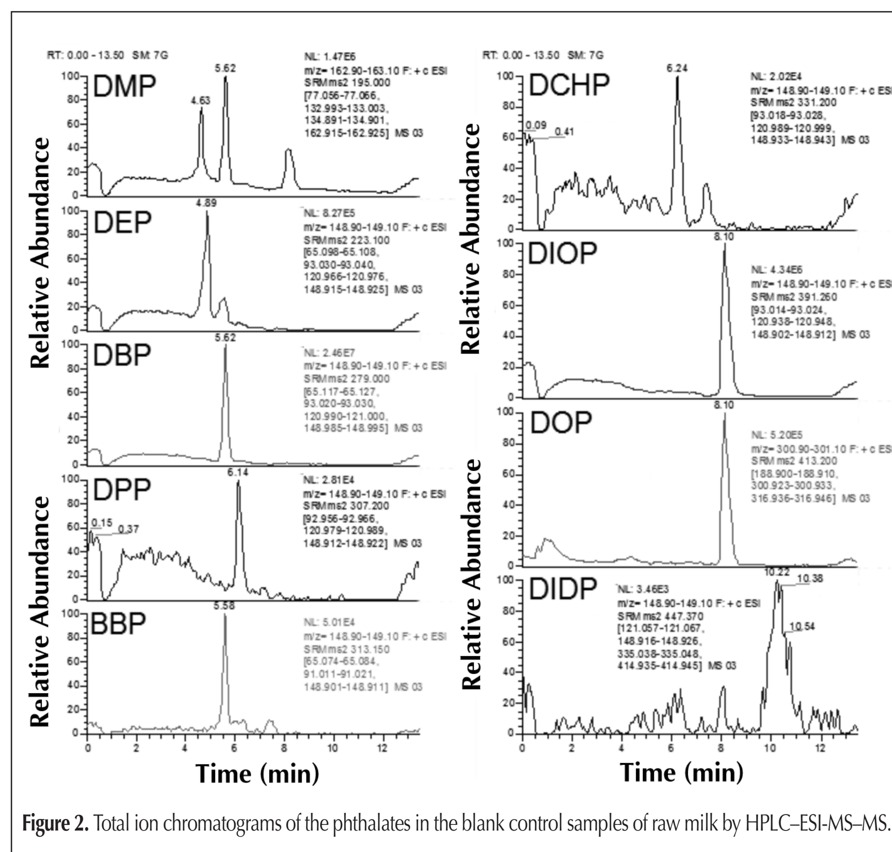

Figure 2. Total ion chromatograms of the phthalates in the blank control samples of raw milk by HPLC–ESI-MS–MS.

more simple and rapid than those reported methods for milk samples (21,22). Due to the complex matrix effects of milk products, proteins must be removed from the samples to increase the extraction efficiency. In our work, good recoveries were obtained following extraction with acetonitrile and sodium chloride. Sodium chloride was introduced to increase the ionic strength of the sample for the sake of removing protein precipitation and enhancing the concentration of the extracted analyte. The addition of sodium chloride was optimized to 0.8 g in order to maximize the extraction recoveries. Because sodium chloride can be dissolved by methanol, it may change the mobile phase of the chromatographic system and influence the chromatographic separation and the ionization. So, the final samples were frozen to remove sodium chloride precipitation before injection. The sonication step can reduce solvent consumption and extraction time, giving an improvement of the whole recoveries for about 5%. The extract could not be filtered. Because the plastic syringes were easy to leach phthalates, especially the DIOP, DOP, and DMP. The membrane also contained phthalates, though it contributed less on the background.

However, the main problem of this sample preparation was the co-extraction of fats. Because lipids have a similar polarity of the phthalates, it was difficult to purify the fat of the milk products with methods based on SPE (1,21). Besides, SPE can lead to more external contamination. The extraction plan with *n*-hexane to remove fats was also impracticable, as it can lead to high background levels of phthalates, especially DIOP and DOP. So the fat was not finally removed from the samples and was directly injected into chromatographic system along with the phthalates. The lipid may adsorb on the chromatographic column and the ion source. Thus, it may influence the separation and ionization of phthalates and reduce the response sensitivity. The mobile phase of 95% methanol maintaining from 3.5 to 11 min can maximize the removal of fat. And then the 20% methanol eluate can maintain the system stable again. The ion source can be cleaned after sampling for 100 times. After all, the preprocessing procedure was very simple, and can minimize the chance of external contamination.

Table IV. Assay Results for Four Commercial Brands of Milk Products (μg/kg)*

Milk	DMP	DEP	DBP	DPP	BBP	DCHP	DIOP	DOP	DIDP
Brand A	62 ± 3.9	–	120 ± 9.2	–	–	46 ± 2.7	71 ± 4.1	–	–
Brand B	57 ± 3.1	31 ± 1.7	127 ± 9.6	29 ± 1.9	–	–	–	84 ± 5.1	–
Brand C	–	35 ± 1.8	113 ± 8.7	38 ± 2.4	24 ± 1.4	–	83 ± 5.3	–	–
Brand D	53 ± 2.9	–	131 ± 9.8	–	–	51 ± 3.2	–	98 ± 6.2	–

* Mean ± SD (n = 6)
– : not detected

The chromatographic and mass conditions were optimize as Table I and II, respectively. The ammonium acetate and formic acid were used as buffer in the mobile phase to improve the chromatography. It was confirmed that the separation was good from the results of Figure 1. The mass analyzer was operated in MS–MS mode. The parent ions $[M+1]^+$ (except DOP $[M+23]^+$) were isolated and specific daughter ions were monitored after further collision. The mass spectrum for all phthalates (except DMP and DOP) were very similar. The main mutual ion was at m/z 149. Two isomeric mixture phthalates (DIOP and DOP) were also successfully analyzed by this proposed method. DOP was monitored as sodiated molecular adduct (M^+Na) instead of M^+H adduct like DIOP. Thus, their fragment ions were completely different. The small additional peak of DIOP in Figure 1 may be the impurities of its standard solution. This can be deduced from the comparison between Figure 1 and Figure 2.

The LOD for these phthalates in this study was comparatively low. The main reasons may be the solvent background and the contamination in the chromatographic system and the complexity of the milk matrix. In fact, it is difficult to get the lower LOD for the ubiquitous contamination of phthalates. The recovery of this method was good using the external standard calibration. It would be better by using the internal standard method, but the isotopically labelled phthalates were not all always commercially available.

Conclusions

In the present study, we have developed a method for the simultaneous determination of nine phthalates in milk using HPLC–ESI-MS–MS. The method is rapid, precise, and accurate. The application of this proposed method to the determination of phthalate residues in milk samples was successful. In short, the analytical method described herein may prove useful for the accurate and highly sensitive detection of phthalate residues.

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