

Original Research

AMMONIUM HYDROXIDE AND MILK FAT CONTENT EFFECT ON BENZYL BUTYL PHTHALATE EXTRACTION

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Abstract

In this study, milk fat content and addition of ammonium hydroxide influence on benzyl butyl phthalate (BBP) determination was investigated. Investigated milk samples contained fat in following amounts (%): 0.5, 1.5, and 3.5. Used method included extraction with hexane with and without addition of ammonium hydroxide. The effects of matrix were studied by spiking milk samples with known amount of BBP standard solution in concentration range 50 to 250 $\mu\text{g L}^{-1}$ before extraction steps. Obtained results indicate that the addition of NH_4OH is proved to be an important procedural step, effectively disrupting matrix bonds in milk samples and increasing BBP recoveries up to five times. While untreated samples showed insufficient recoveries (below 50%), the alkaline modification achieved reliable results across all fat levels, even reaching over 100% in specific samples. these results highlight the important role of alkaline conditions in disrupting milk's complex matrix and facilitating the release of hydrophobic phthalates such as BBP.

Keywords: milk, fat content, phthalate, ammonium hydroxide.

INTRODUCTION

Phthalates are chemical substances widely used as additives in production of plastic materials to increase softness and flexibility [1,2]. Bearing in mind a large amount of plastic production, phthalates can be found in many consumer products, such as medical equipment, cosmetics, clothing, as well as food packaging, etc. Consequently, humans may be exposed to phthalates through ingestion, dermal absorption, and inhalation, with food ingestion considered the primary route of exposure [3,4]. Phthalates do not form stable bonds with plastics and can release into surrounding media, and food can be contaminated by the contact with food packaging containing phthalates.

Phthalates have lipophilic nature and tend to leak from plastics in oil or fat-containing food [1,5]. In recent years, phthalates have attracted huge public attention and are listed as

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contaminants of emerging concern because they are recognized as endocrine disruptors or mutagens, which can have adverse effects on human health even at low levels [6].

Milk is a complex matrix consisting of proteins and different amounts of fat, and phthalates can be bound to matrix constituents with different affinity due to their different chain lengths. Milk and milk products can be contaminated during the preparation and packaged processes. The tolerable daily intake (TDI) values established by the European Food Safety Authority (EFSA) panel is 0.5 mg/kg/day for benzyl butyl phthalate (BBP) [6,7].

MATERIALS AND METHODS

Chemicals

BzBP, dibutyl adipate (DBA) and hexane were purchased from Sigma Aldrich, USA. Hexane was HPLC grade and screened to determine the phthalate background. Stock solution of phthalate and DBA standards were prepared by hexane diluting the amounts that were accurately weighted out by analytical balance with precision at ± 0.00001 g (Kern, Germany) and stored at 4 °C. Working standard solutions were at the concentration range from 0.25 to 10 $\mu\text{g mL}^{-1}$. DBA was used as internal standard at concentration 1 $\mu\text{g mL}^{-1}$.

All glassware used in this study was soaked and washed with acetone, rinsed with hexane and dried at 120 °C for 4 h to avoid phthalate contamination. After cleaning, glassware was sealed with aluminium foil and stored in a clean environment to avoid adsorption of phthalates from the air.

Samples

Milk samples were purchased from local supermarkets (Niš) and all samples were open at the same time in the same place. Three different types of milk with different milk fat content (0.5, 1.5 and 3.5%) were investigated. The effects of matrix were studied by spiking samples with known amount of phthalates standard solution in concentration range 50 to 250 $\mu\text{g L}^{-1}$ before extraction steps. All spiked samples and blank sample were analysed in three replicates.

A volume of 10 mL spiked milk sample was put in a polypropylene centrifuge tube and 0.5 mL of 3% NH_4OH was added. Mixture was vortexed for 1 min at 1500 rpm (Vortex Genie 2, Scientific Industries, USA) and 30 min at room temperature, to denature proteins. After this step, 5 mL of hexane was added, and mixture was vortexed 2 min at 2000 rpm, and centrifuged 10 min at 4000 rpm (Jouan C4i centrifuge, Thermo Scientific). About 5 mL of supernatant was transferred into clean glass tube, evaporated near to dryness at 30 °C, and reconstituted by hexane. Samples were vortexed 1 min at 1000 rpm and transferred to glass vial. DBA was added and samples were analysed by gas chromatography-mass spectrometry. All spiked samples were analysed in three replicates. The same procedure was repeated for spiked milk samples without NH_4OH addition.

Instruments

Analysis was carried out by gas chromatography coupled to mass spectrometer (Hewlett Packard 6890 series GC System with autosampler connected with Agilent 5973 Mass Selective Detector (Electron Ionization MSD-EI, single quadrupole). The separation was achieved with 30 m $\times$ 0.25 mm $\times$ 0.25 μm a non-polar AGILENT DB-5MS column coated

with 5% phenyl, 95% dimethylpolysiloxane. The MSD was used in the single ion-monitoring (SIM) mode. The identification of target compounds was based on the relative retention time, the presence of target ions and their relative abundance. The most abundant ion m/z 149 was chosen for quantification of BBP. Ion m/z 185 was chosen as representative ion of DBA internal standard. The dwell time was 100 ms.

RESULTS AND DISCUSSION

Calibration curve of BBP standard series was obtained by standard solutions in a concentration range of 0.25 to 10 $\mu\text{g mL}^{-1}$. Obtained calibration curve was linear with correlation coefficient > 0.9982 . Recovery values were calculated for all investigated samples, and their mean values are given in Table 1 and 2.

The obtained data demonstrate a pronounced influence of NH_4OH addition on the extraction efficiency of BBP from milk samples. Without NH_4OH , recoveries were consistently low across all tested concentrations, generally remaining below 50%. This pattern was observed regardless of milk fat content, although samples with 1.5% fat showed slightly higher recoveries compared to 0.5% and 2.5% fat. Such limited extraction efficiency suggests that the native matrix environment of milk—particularly its protein–lipid interactions—restricts the release of BBP during sample preparation.

In contrast, the introduction of NH_4OH significantly enhanced BBP recovery, indicating a strong matrix-modifying effect. Recoveries increased two- to five-fold depending on fat content and spiking level. The most notable improvements were observed in samples with 1.5% fat, where recoveries exceeded 100% at certain concentrations, suggesting both improved extraction and potential matrix-induced signal enhancement. Even in higher-fat samples (2.5%), where analyte entrapment is typically more pronounced, NH_4OH addition resulted in recoveries around 60–123%, representing a substantial improvement over the untreated samples.

Table 1 Recovery values obtained for BBP extraction from milk samples without NH_4OH addition

Phthalate	Added concentration (ng g^{-1})	Milk fat content (%)		
		0.5	1.5	2.5
BzBP	5	21.56 $\pm$ 0.69	42.97 $\pm$ 2.01	24.05 $\pm$ 1.00
	15	19.60 $\pm$ 0.35	48.45 $\pm$ 1.98	45.71 $\pm$ 1.15
	25	18.48 $\pm$ 0.45	47.61 $\pm$ 1.78	44.35 $\pm$ 1.08

Table 2 Recovery values obtained for BBP extraction from milk samples with NH_4OH addition

Phthalate	Added concentration (ng g^{-1})	Milk fat content (%)		
		0.5	1.5	2.5
BzBP	5	73.26 $\pm$ 3.09	104.71 $\pm$ 4.28	59.94 $\pm$ 1.29
	15	93.24 $\pm$ 3.03	125.42 $\pm$ 3.05	123.58 $\pm$ 2.04
	25	77.45 $\pm$ 3.58	63.24 $\pm$ 2.12	63.42 $\pm$ 1.21

Overall, these results highlight the critical role of alkaline conditions in disrupting milk's complex matrix and facilitating the release of hydrophobic phthalates such as BBP. The comparison clearly demonstrates that NH_4OH addition is essential for achieving reliable and

reproducible extraction performance across varying fat levels. This finding is particularly relevant for analytical workflows targeting phthalates in dairy products, where matrix effects can otherwise compromise quantification accuracy.

CONCLUSION

This study demonstrates that the complex matrix of milk, characterized by strong protein–lipid interactions, significantly disturbs the extraction of benzyl butyl phthalate (BBP). The addition of NH_4OH proved to be an important procedural step, effectively disrupting these matrix bonds and increasing BBP recoveries up to five times. While untreated samples showed insufficient recoveries (below 50%), the alkaline modification achieved reliable results across all fat levels, even reaching over 100% in specific samples. These findings indicate that NH_4OH not only facilitates analyte release but also potentially mitigates matrix-induced signal suppression. This optimized approach provides a robust and reproducible framework for the accurate quantification of hydrophobic phthalates in dairy products. This kind of methodological improvements are essential for ensuring food safety and conducting reliable exposure assessments in complex food matrices.

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