Extraction of Phthalates in Commercial Milk Products Using ISOLUTE® SLE+ Supported Liquid Extraction Columns Prior to LC-(+) APCI-MS/MS Analysis

This application note describes a novel sample cleanup strategy to address the complex matrix effects associated with commercial milk product variables while maintaining adequate analyte recovery and repeatability

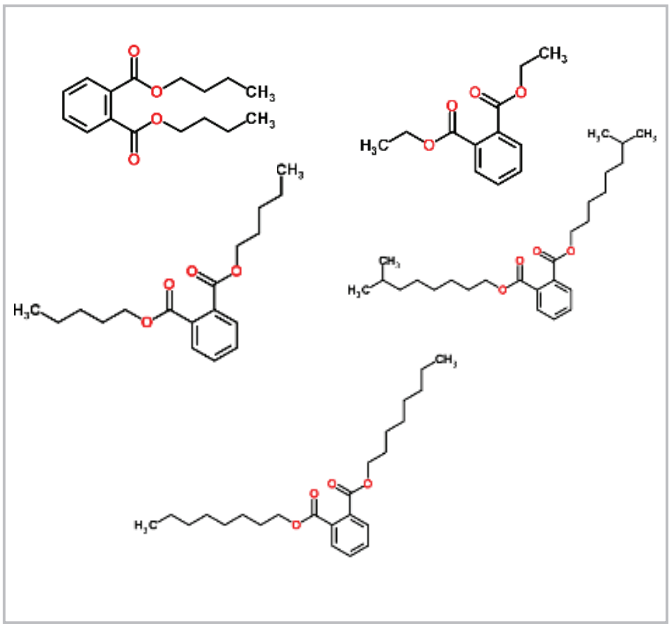

Figure 1. Structures of the selected phthalate analytes

Introduction

The migration of plastizers such as phthalic acid esters (phthalates) from the surface of food packaging materials to processed food materials and subsequent fate in man has long been an issue of public health. Continued interest in the biomonitoring of these compounds has inspired a number of method development strategies, however, classic methods are labor intensive and require multi-step time consuming efforts. For this reason, a novel method has been developed. The method development path for the determination of phthalates in commercial milk products was demonstrated using a 3 stage sample preparation workflow prior to gradient LC-(+)APCI-MS/MS. A set of milk samples fortified with 5 phthalates was diluted with isopropanol (IPA) and processed using an automated bead mixer to disrupt nonselective binding. The samples were then centrifuged and loaded onto a supported liquid extraction single use cartridge. This method was applied to commercial milk product variables to study the effect of fat content on relative recovery.

ISOLUTE® SLE+ Supported Liquid Extraction plates and columns offer an efficient alternative to traditional liquid-liquid extraction (LLE) for bioanalytical sample preparation, providing high analyte recoveries, no emulsion formation, and significantly reduced sample preparation time.

Analyte

Diethyl phthalate, dibutyl phthalate, dipentyl phthalate, dioctyl phthalate, di-iso-nonyl phthalate

Sample Preparation Workflow: Mix, Centrifuge and Extract

Format:	ISOLUTE SLE+ 1 mL Sample Volume Columns (Tabless), part number 820-0140-CG
Bead Mix:	Samples were prepared by adding 1 mL of milk and 2 mL of IPA to a 7 mL vial containing 8 ceramic beads. The samples were then loaded onto the BeadRuptor 24 (OMNI International, Atlanta, GA). Samples were spun at 6 M/s for 25 seconds. The cycle repeated for a total of 2 cycles (0.05 dwell pause between cycles).
Centrifuge:	The mixed samples were transferred (3 x 1 mL) to clean / dry centrifuge tubes using 1 mL Eppendorf pipette tips (leaving the beads). The samples were centrifuged for 3 min at 2900 rpm to exaggerate the density gradient, and crash proteins and syrup components (when applicable).
Extract:	The crashed samples were further processed by loading 1 mL of the supernatant on an ISOLUTE SLE+ column. The mixture was allowed to equilibrate on the column for 5 min. Analytes were eluted using a two-step elution strategy: hexane (3 mL), followed by dichloromethane (CH_2Cl_2) (3 mL).
Evaporation:	The combined extracts were then evaporated using a TurboVap LV. Extracts were kept under N2 for an additional 5 min after tubes appeared empty.
Post Extraction:	The samples were then reconstituted with acetonitrile (1 mL).

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HPLC Conditions

Instrument: Agilent 1200 Liquid Handling System (Agilent Technologies, Berkshire, UK)

Column: Biotage Resolux C4 column (2.1 mm x 150 mm, 4.5 µm) (catalog # R2-1521-2045)

Mobile Phase: A: 10mM Ammonium Acetate
B: Acetonitrile

Gradient:

Table 1. Linear gradient chromatography conditions

Step	Time (min)	Flow Rate (µL/min)	%A	%B
1	0.0	1000	100	0
2	0.50	1000	100	0
3	4.5	1000	0	100
4	7.0	1000	0	100
5	7.5	1000	100	0
6	11.0	1000	100	0

Injection Volume: 5 µL

Temperature: 40 °C

Mass Spectrometry Conditions

Instrument: Applied Biosystems/MDS Sciex 4000 Q-Trap triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA.) The APCI probe was used for this analysis.

Ion Source Temperature: 700 °C

Nebulizer Current (NC): 5 °C

Table 2. MRM transitions for phthalates in positive mode APCI-MS/MS

Scan Function	Analyte	MRM Transition	Declustering Potential (DP)	Collision Energy (CE)	Cell Exit Potential (CXP)
1	Diethyl phthalate	223 → 177/149	54	15	16
2	Dibutyl phthalate	279 → 205/149	54	18	16
3	Dipentyl phthalate	307 → 219/149	54	35	16
4	Dioctyl phthalate	391 → 261/149	54	20	16
5	Di-iso-nonyl phthalate	419 → 275/149	54	20	16

Reagents

HPLC grade water, acetonitrile, n-propanol, dichloromethane and hexane were purchased from Sigma-Aldrich Co. (Atlanta, GA.). The phthalate standards (also purchased from Sigma-Aldrich Co.) are detailed in **Table 3**.

Table 3. Identification of the selected phthalate analytes

Name	Abreviation	CAS No.	Log P
Diethyl phthalate	DEP	84-66-2	2.71
Dibutyl phthalate	DBP	84-74-2	4.75
Dipentyl phthalate	DPP	131-18-0	5.77
Di-n-octyl phthalate	DNOP	117-84-0	8.83
Di-iso-nonyl phthalate	DINP	68515-48-0	9.77

Results

The three commercial milk products were purchased from local grocery stores and tested the same day as purchased. The milk samples were verified within the designated shelf life. These commercial milk samples were fortified with a working standard solution containing the 5 phthalate analytes. The concentration of these analytes ranged from 1-5 µg/mL. The method performance was evaluated based on relative recovery and repeatability. Three commercial milk variables demonstrated typical relative recoveries from ~80-120% for diethyl-, dibutyl- , dipentyl-, dioctyl- and di-iso-nonyl-phthalate (**Figure 2**). This method demonstrated typical repeatability (%RSD) for n = 7 replicates ‹ 20%.

It was determined that diluting the isopropanol (IPA) supernatant with water prior to loading the sample on the cartridge caused a significant decrease in relative recovery for diethyl phthalate. For this reason, the centrifuge supernatant was loaded directly onto the cartridge. The 2 stage hexane and dichloromethane elution strategy was required to maintain adequate relative recovery for all of the selected analytes. Note: If either elution solvent was applied (2 x 3 mL) without the other, the relative recovery was more variable and less robust.

The bead mixer proved an interesting tool in this study as the fundamentals of the observed benefit are unknown at this time. It was observed that acetonitrile did not perform as well as IPA as a crash solvent. The IPA crash without the bead mixer in the workflow provided relative recoveries ‹50% for dipentyl-, dioctyl- and di-iso-nonyl-phthalate. It was observed that the 7 mL tubes after the bead mixing cycles were warm. The bead mixer outperformed sonication with and without pH modifiers on merits of data quality and analysis time.

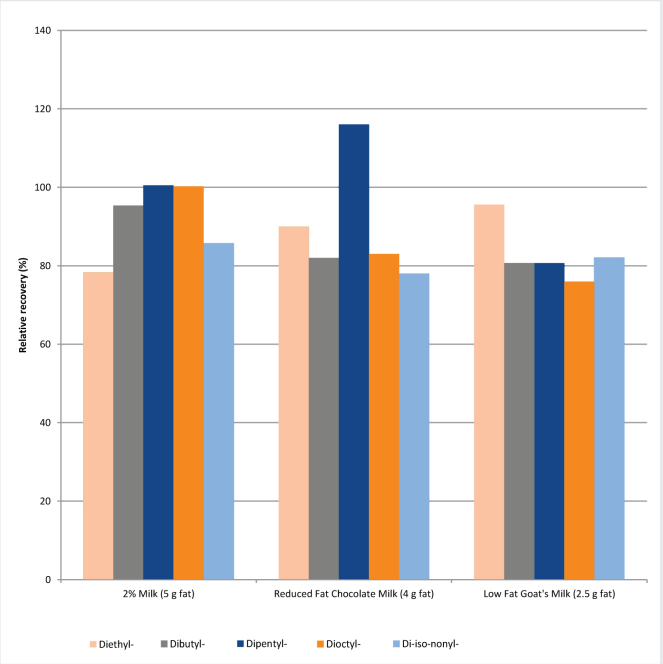

Figure 2. Relative recovery (%) for 5 phthalate analytes in 3 commercial milk variables

Figure 3. Bead Ruptor 24

Acknowledgements:

Biotage would like to thank Pete Tortorelli and James Atwood with OMNI, International for providing the Bead Ruptor24 for this study. OMNI International, 935-C Cobb Place Boulevard Kennesaw, GA 30144 United States, http://www.omni-inc.com/

Ordering Information

Part Number	Description	Quantity
820-0140-CG	ISOLUTE® SLE+ 1 mL Sample Volume Columns (Tabless)	30
C103198	TurboVap LV 120V	1
C103199	TurboVap LV 220V	1
PPM-48	Biotage® PRESSURE+ 48 positive pressure manifold 48 position	1
R2-1521-2045	Resolux 200, C4 LC column, 150 x 2.1 x 4.5	1

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