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# Contemporary carbon content of bis (2-ethylhexyl) phthalate in butter

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ABSTRACT

The fraction of naturally produced bis (2-ethylhexyl) phthalate (DEHP), a ubiquitous plasticizer known to contaminate packaged foods, was determined for each of five 1.10 kg samples of unsalted market butter by accelerator mass spectrometry (AMS). After extraction and concentration enrichment with liquid–liquid extraction, flash column chromatography, and preparative-scale high performance liquid chromatography, each sample provided ≈250 μg extracts of DEHP with carbon purity ranging from 92.5 ± 1.2% ($n$ = 3, 1σ) to 97.1 ± 0.8% ($n$ = 3, 1σ) as measured with gas chromatography mass spectrometry (GC–MS). After corrections for method blank DEHP, co-eluting compounds, and unidentified carbon, the mean fraction of naturally produced DEHP in butter was determined to be 0.16 ± 0.12 ($n$ = 5, 1σ). To our knowledge, this is the first report of the contemporary fraction of DEHP isolated from market butter in the U.S.
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## 1. Introduction

With an annual worldwide production of 1–4 million metric tons (Pocar et al., 2012), bis (2-ethylhexyl) phthalate (DEHP) is the most widely used commercial plasticizer. It is commonly used as a softener to improve material flexibility in plastic pipes (up to 30% in PVC by mass) (European Chemicals Bureau, 2008), tubing (including those used in medical procedures), packing materials (including those used in food packaging) (Rudel et al., 2011); and as a thickener in cosmetics, personal care products (Koo & Lee, 2004), and printing inks (including those used in food-wrap labels) (Nerin, Cacho, & Gancedo, 1993). DEHP is a viscous liquid at room temperature and is not covalently bonded to polymeric matrices. Therefore it readily diffuses from plastics and various other products into blood used in transfusions, food, and drinks (Rais-Bahrami, Nunez, Revenis, Luban, & Short, 2004). Approximately 2% of the global phthalate production is released into the environment each year and part of this release is incorporated into the food chain (Huber, Grasl-Kraupp, & Schulte-Hermann, 1996).

In addition to its anthropogenic production, algae and certain microorganisms synthesize DEHP naturally. Chen (2004) found that the red alga, *Bangia atropurpurea*, from shallow costal waters of Taiwan, synthesized DEHP, *de novo*, as evidenced by cultivating it in the laboratory with $NaH^{14}CO_3$ (Chen, 2004). Japanese scientists, Hayashi, Asakawa, Ishida, and Matsuura (1967) found phthalates in *Cryptotaenia canadensis DC. Var. Jayonic Makino*, a perennial vegetable cultivated in Japan, albeit the sources of these phthalates were not well established (Hayashi et al., 1967). Some fungi also synthesize DEHP. For example, *Penicillium olsonii* produces DEHP as a metabolite (Amade, Mallea, & Bouaicha, 1994). As algae and other microbes are widely used in herd forage, incorporation into dairy products is possible.

DEHP has a low acute toxicity and can be metabolized quickly in humans. In fact, 47% of the DEHP ingested is excreted in urine after metabolic hydroxylation and hydrolysis to produce mono (2-ethyl-5-hydroxyhexyl) phthalate, mono (2-ethyl-5-oxohexyl) phthalate, and mono (2-ethylhexyl) phthalate (MEHP), within two days after ingestion (Koch, Bolt, & Angerer, 2004). Nevertheless, evidence suggests that both high-level acute and chronic exposures to DEHP (up to 0.05% of diet mass) have induced hepatic tumorigenesis in mice (Ito et al., 2007), infertility by endocrine function disruption in female rats (1.4 g $kg^{-1}$ body mass, twice per week for 26 weeks) (Hirosawa, Yano, Suzuki, & Sakamoto, 2006), and male feminization in humans (Lottrup et al., 2006).

According to the U.S. Department of Health and Human Services (DHHS), prior to 2002, inhalation of contaminated indoor air, ingestion of contaminated water and food, and exposure to DEHP from plastic medical products were the dominant pathways leading to human exposure of DEHP (Agency for Toxic Substances & Disease Registry, 2002). Although the usage of DEHP has been voluntarily reduced in food contact materials, phthalate esters are still

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found in foods (Alderson, 2008). Prior to 2002, the daily DEHP intake from food in the U.S. was reported to be 5.8 mg (Giust, Seipelt, Anderson, Deis, & Hinders, 1990). In 2011, DHHS reported daily DEHP intake (1–30 μg/kg body weight) equivalent to be at most 2.2 mg (Department of Health, 2011). Despite this reduction in dose, the issue of phthalates in food matrices remains of concern to the US-FDA, provided that naturally occurring biochemical processes do not synthesize the phthalates in food.

Carbon in compounds synthesized by the biogenic processes of living organisms contain approximately one part per trillion of radiocarbon ($^{14}C$) owing to incorporation of $^{14}CO_2$ formed in the upper-atmosphere by oxidation of $^{14}C$ produced by cosmic neutron reactions on $^{14}N$. $^{14}C$ decays with a half-life of 5730 ± 40 years (Cambridge half-life, 1962). As virtually all commercially-produced DEHP is made from petrochemical feed-stocks that have been isolated from the atmosphere for millions of years, petrochemically derived DEHP contains $^{14}C/C < 10^{-15}$. As demonstrated by Eglinton et al. (1997), the abundance of $^{14}C$ in individual compounds in complex matrices can be determined by compound-specific accelerator mass spectrometry (CS-AMS) (Eglinton et al., 1997).

CS-AMS has been applied to DEHP isolated from three strains of algae: *Undaria pinnatifida*, *Laminaria japonica* and *Ulva* sp. (Namikoshi, Fujiwara, Nishikawa, & Ukai, 2006), and more recently DEHP isolated from Stilton Cheese, a cheese injected with *Penicillium* (Nelson, Ondov, VanDerveer, & Buchholz, 2013). Results from the former suggest that large fractions of the DEHP extracted from algae (49.8–87.2%) were synthesized by natural biogenic processes. The latter suggests that a significant fraction (23.5 ± 7.3%) of the DEHP in Stilton Cheese could be attributable to natural biogenic processes, possibly owing to production by *Penicillium* sp.

Butter contains the highest mean level of lipids (81.1% w/w) in dairy products (U.S. Department of Agriculture, 2013). DEHP concentrations in butter as great as 2.4 mg/kg have been reported (Sharman, Read, Castle, & Gilbert, 1994). By our own analyses, the average DEHP content in recently purchased Giant-brand unsalted butter was 0.70 ± 0.03 mg/kg ($n = 4$, $1\sigma$). Since butter is neither fermented nor contains mold, bacteria, or algae, the natural abundance of $^{14}C$ in its DEHP could be expected to be less than that found in Stilton cheese. For these reasons, and to further develop the data available for the origin of DEHP in fatty food, CS-AMS was applied to DEHP isolated from several batches of market butter.

## 2. Methods

### 2.1. Extraction and enrichment

Five 1.10-kg batches of butter (Giant, unsalted, distributed by Foodhold USA, Inc., Landover, MD 20785) were extracted with hexane and purified with flash chromatography and preparative-scale HPLC to obtain ≈250 μg DEHP per batch. Three method blanks were simultaneously prepared to determine DEHP contamination resulting from the solvents, column packings, and contact with surfaces of the apparatuses. Approximately 570 μg of fully deuterated *d38*-DEHP (98% pure, Cambridge Isotope Laboratories, Andover, MA) were spiked into both butter batches and method blanks at the very beginning to determine the yields and to assist identification of peaks in the HPLC chromatograms.

For each batch, the butter was dissolved in 1.0 L hexane (J.T. Baker, 95% n-Hexane) with gentle heat (≈40 °C). The supernatant fluid was removed by gravity filtration. The material remaining insoluble was then extracted with 300 mL of 17% v/v acetone (Sigma Aldrich, ACS reagent, ⩾99.5%) in hexane and filtered. Both of these filtered extracts were combined and reduced to 1200 mL with rotary evaporation. DEHP is nearly 7-fold more soluble in acetonitrile than saturated fatty acid esters (Kotowska, Garbowska, & Isidorov, 2006; Zhou, Chen, & Li, 2002). Therefore, each 400 mL extract was mixed with 1.0 L acetonitrile (J.T. Baker, HPLC grade, 99.9%) for solvent–solvent partitioning. After partitioning the resulting 3-L, light-yellow, DEHP-enriched-acetonitrile layer was reduced to 1.0 L and stored in a freezer (−20 °C) for 12 h. Lipids and proteins precipitated after cooling and were removed by gravity filtration. Solvent was removed from the filtrate by rotary evaporation and the residual was retrieved in 4 mL hexane for subsequent purification. Each butter extract was split into four 1-mL fractions for processing on a newly-packed flash column with silica gel (32–63 μm, Dynamic Adsorbents, Atlanta, GA) and eluted with a 1.6% v/v mixture of acetone in hexane under pressure of charcoal-scrubbed (model 300 high-pressure hydrocarbon trap, Chromatography Research Supplies, Louisville, KY) and filtered (Swagelok stainless steel in-line particle filter) compressed air. The DEHP fraction was collected between 1100 mL and 1500 mL of the mobile phase, as verified by gas chromatography-electron-impact-mass spectrometry (GC–EIMS) (Shimadzu® QP2010S, Shimadzu® SHRXI-5MS column, 30 m, 0.25 μS I.D., polysiloxane coated). All DEHP-containing eluates were combined to yield 1600 mL (per 1.1 kg butter sample) of hexane solution, dried, and re-dissolved in 1 mL of acetonitrile for preparative-scale HPLC.

A Hewlett–Packard series 1050 HPLC with a C18 column (15 cm × 9.4 mm-ID, Agilent® Zorbax Eclipse) was used for further purification. To achieve a reasonable resolution ($R = 1.1$) between d38-DEHP and DEHP, gradient elution was applied starting with 90% acetonitrile and 10% water at 30 °C at 4 mL/min. After 10 min, the mobile phase composition was adjusted to 95% acetonitrile and 5% water, and elution continued at the same temperature and flow rate. The instrument's diode array detector (DAD) was set to 254 nm, the maximum absorbance of DEHP (Orsi et al., 2006). *d38*-DEHP and DEHP were eluted at 19 and 21 min, respectively. Eluates were collected in aluminum-foil wrapped vials (pre-baked at 425 °C for 12 h). Approximately 50 mL of DEHP-containing eluate (95% acetonitrile, 5% water) were collected per each 1.1 kg butter sample. Prior to further analyses, the volume of each eluate was reduced to 1 mL by rotary evaporation and the residual again dissolved in 2 mL of hexane. Method blanks were prepared using this same set of procedures.

Prior to AMS, the carbon mass and purity of DEHP in each of the (2-mL, in hexane) butter sample extracts was determined by GC–EIMS with a series of standard petrogenic DEHP (99.8 ± 0.1% pure, Supelco® Analytical, Bellefonte, PA) solutions. GC–EIMS purity was determined from peak areas of the total ion chromatogram, and by three-dimensional deconvolution within the DEHP peak to determine co-eluting compounds (see Table 1). These analyses were performed with a 1-μL injection, 1.00 mL/min column flow of helium, initial temperature of 90 °C, increasing by 15 °C/min for 20 min. EIMS ion spectra were collected from 50 to 500 $m/z$ at a scan rate of 3.3 scans $s^{-1}$. Up to 21 co-eluted compounds were identified in the GC–EIMS spectra by matching against the NIST GC–EIMS standard spectral library. On average, three compounds, Z, E-2, 13 octadecadien-1-ol, cholesterol, and siloxanes, accounted for approximately 17%, 20% and 58%, respectively, of the total co-eluted carbon mass. Siloxanes observed were consistent with GC-column bleeding and were, therefore, not included in purity and mass determinations. The apparent carbon fraction of DEHP (carbon purity), carbon mass in DEHP ($m_{DEHP}$) and method blank ($m_{DEHP,mtd}$), and mass of carbon in identified coeluting compounds ($m_{icoe}$) were determined from the GC–EIMS spectra. These are listed in Table 1 for each isolate sample to facilitate $^{14}C$ corrections described below.

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