CONTEMPORARY FRACTION OF BIS(2-ETHYLHEXYL) PHTHALATE IN STILTON CHEESE BY ACCELERATOR MASS SPECTROMETRY

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ABSTRACT. Measurements of the radiocarbon abundance in 5 samples of bis(2-ethylhexyl) phthalate (DEHP) isolated from Stilton cheese were made by accelerator mass spectrometry (AMS) to determine the fraction of carbon originating from contemporary biogenic sources. DEHP is classified as a "priority hazardous substance" by the European Union, a probable human carcinogen by the United States Environmental Protection Agency, and is suspected to be a human endocrine disrupter. Measurement of its ^{14}C abundance in a specific food indicates whether its presence is due to contamination from industrially synthesized DEHP or a naturally inherent component. A method was developed to determine the contemporary carbon fraction of DEHP in a fatty food matrix at concentrations of ≈0.14 mg/kg. Five 90-µg quantities of DEHP were extracted from 12 kg of Stilton cheese and isolated by silica gel, size exclusion, and high-performance liquid chromatography (HPLC). Masses of samples were determined by gas chromatography mass spectrometry (GC-MS) analyses prior to combustion and manometry afterwards. The purity of DEHP carbon mass in each isolate was determined by multivariate deconvolution of GCMS fragmentation spectra obtained from measurements of standards and isolates, and ranged from 88.0 ± 1.8% to 92.3 ± 1.1% (n = 5, 1σ). Concurrently processed isolation method blanks contained from 0.15 ± 0.04 to 1.52 ± 0.06 µg (n = 3, 1σ) DEHP per sample and significant quantities of pre- and post-chromatographic extraneous carbon contamination. The mean ^{14}C-corrected contemporary carbon fraction of DEHP in the isolates was 0.235 ± 0.073 (1σ; and ± 0.091 at the 95% confidence level), revealing that the majority of DEHP in Stilton cheese results from anthropogenic sources, but with a significant naturally occurring component.

INTRODUCTION

Phthalic acid esters (PAE, i.e. 1,2-benzenedicarboxylic acids), also known as phthalates, are used in the manufacturing of many consumer and industrial products including pharmaceuticals, plastic polymers, adhesives, inks, and cosmetics (Schettler 2006). Bis(2-ethylhexyl) phthalate (DEHP) comprises half of the estimated 8 million tonne annual industrial phthalate production (Wenzl 2009). Leaching of this compound from many plastic consumer products ultimately leads to its environmental ubiquity and exposure (Schettler 2006).

Despite its low acute toxicity, DEHP has been classified a probable human carcinogen (US EPA 1997) and is suspected to be a human endocrine disruptor that mimics estradiol, causing irregular development and feminization in young boys (Jarosova 2006) and possibly decreased fertility in men and in women of child-bearing age (Zhu et al. 2006; David 2000). Accordingly, some developed nations, including the US and those in the European Union, have phased out the use of phthalates in food-contact materials (Enneking 2006) due to their proclivity for leaching into fatty foods (Castle et al. 1990). Human dietary consumption has been identified as a significant source of DEHP (Wormuth et al. 2006) and possibly the single most likely route of exposure to the general populace (Fromme et al. 2004; Schettler 2006). Unlike many other phthalate-containing materials, such as cosmetics and certain plastic products, the consumption of food is a necessary and unavoidable route to human exposure.

A comprehensive European study of DEHP in food reports that concentrations in non-dairy beverages averaged 0.01–0.04 mg/kg; non-fatty foods such as fruit, vegetables, and grain products, 0.01–

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0.57 mg/kg; and fatty foods such as oils, dairy, animal, and nut products, 0.22–1.45 mg/kg (Wormuth et al. 2006). These concentrations were consistently greater than those observed in 6 other phthalates in the same food matrices, which is not surprising considering the much larger industrial production of DEHP. These values support the contention that phthalates are typically more concentrated in foods with a high lipid component and that DEHP is the most abundant of these compounds in commonly consumed foods. Screening analyses performed in our laboratories prior to work described herein found DEHP to account for most of the PAE content in various cheeses at mass fractions of 0.20–0.41 mg/kg (M Nelson, unpublished data).

Although contamination by anthropogenic phthalates may continue to occur, evidence of naturally produced phthalates has been reported for several types of algae and fungus. Chen (2004) demonstrated the biosynthesis of DEHP by red algae (*Bangia atropurpurea*) grown in a culture medium containing ^{14}C-labeled sodium bicarbonate. More recently, Namikoshi et al. (2006) determined the modern carbon content of DEHP isolated from 3 different algae (2 of which are edible) to range from 50% to 87%. Previously, Amade et al. (1994) had determined that DEHP comprised 23% of the ethanol-extracted compounds from fungal culture broths of *Penicillium olsonii*, and suggested it to be a metabolite of the fungus. Many algae products are used as food additives, and penicillium is often used as a fermentation agent in foods such as blue cheese. That DEHP can be synthesized by certain types of algae and mold used in food suggests that they are potentially important additional sources of dietary DEHP exposure, especially in light of the reduction of DEHP in food-contact materials. Therefore, the United States Food and Drug Administration (US FDA) is interested in determining the origin of DEHP in food, particularly fatty foods.

Synthesis of DEHP by biological sources likely amalgamates atmospheric steady-state ^{14}C from the biological origins of the food matrix from which it is produced. Petroleum formed from organic matter that has been isolated from atmospheric $^{14}CO_2$ for millions of years has a ^{14}C:^{12}C ratio that has decayed to immeasurably low levels (Namikoshi et al. 2006). Synthetic, anthropogenic DEHP used in plastics is produced from a 2-step esterification of petrogenic phthalic anhydride with petrogenic ethylhexanol (ECPI 2012), and thus is also devoid of measurable ^{14}C. Therefore, measurements of ^{14}C:^{12}C in DEHP content in food will reveal whether it is naturally produced by biosynthesis from contemporary carbon sources or if more can be done to reduce anthropogenic environmental and food-processing contamination.

Compound-specific radiocarbon analysis (CSRA) has been performed to determine the age and origin of <100-µg quantities of compounds similar to DEHP, including polycyclic aromatic hydrocarbons (PAH) ranging in concentration from 0.03 to 10 mg/kg in several environmental Standard Reference Materials (Reddy et al. 2002) and lipid biomarkers at ≥1 mg/kg concentrations (Pearson et al. 2001) in marine sediments. There have been no ^{14}C measurements of phthalates in processed food and, most notably, none in fatty foods. It was a goal of the US FDA to obtain ^{14}C data on food matrices. For this purpose, Stilton cheese was selected for purification and ^{14}C analyses. Stilton cheese was selected due to its high fat content (35% mass fraction) and because Stilton cheese, like other blue Roquefort cheeses, is injected with cultures of *Penicillium roqueforti*, which might also be expected to synthesize DEHP from contemporary compounds in the cheese.

Although single compound analyses of as little as 10 µg of carbon have been reported, and the Lawrence Livermore National Laboratory Center for Accelerator Mass Spectrometry (LLNL CAMS) routinely performs analyses on samples as small as 20 µg carbon, 100-µg masses are preferable when supply of the sample is not a limiting factor. Accordingly, we sought to develop a method to isolate 70–100 µg quantities of DEHP. As the Stilton cheese analyzed in our work con-

tained as little as 0.12 mg/kg DEHP, a 7.5-million-fold enrichment was required to obtain these quantities at 90% purity.

METHODS

Samples and Extraction

Two 7-kg wheels of Stilton cheese (Neal's Yard Borough Market brand, London, UK) were purchased from a Whole Foods Market (Gaithersburg, MD, USA) and received in the manufacturer's original paper packaging for use in this study. Twelve kilograms of cheese was extracted in seven 1.2–2.6 kg batches to isolate enough DEHP to provide five 70- to 100-µg samples of carbon for analysis by accelerator mass spectrometry (AMS). In addition, 4 method blanks were prepared contemporaneously (see Table 1) using only the extractants, eluents, and diluents used in the sample extraction-purification procedure. A fully deuterated (*d38*) DEHP phthalate standard (98% pure, Cambridge Isotope Laboratories, Andover, MA, USA) was used to spike the raw cheese matrix in order to determine method yield and act as a resolvable chromatographic reference for unlabeled DEHP during the purification steps. The 4 method blanks were likewise spiked. Cheese samples were cut vertically from the wheels to ensure uniformity of the sampling location, homogenized, and sequentially extracted 3 times by manually stirring with 3 different solvents at 40 °C (2400 mL hexanes; 1200 mL 5:1 volume fraction hexane:acetone; and 800 mL hexanes). The 3 extracts were combined, evaporated to 2 L, and centrifuged at 66,850 rad/s for 10 min to remove solids.

DEHP has been shown to be more soluble in acetonitrile than in non-polar fats in the cheese matrix (Castle et al. 1990). Accordingly, each hexane extract was partitioned into 3.3 L of hexane-saturated acetonitrile, the acetonitrile phase separated, evaporated to 2 L, and stored at –20 °C. After chilling, the acetonitrile extract was gravity-filtered while still cold to remove protein and fat precipitates. The DEHP in these extracts and contemporaneously processed blanks was further purified using a series of 3 chromatographic separations: silica-gel "flash" chromatography (FC); size exclusion chromatography (SEC); and reverse-phase high-performance liquid chromatography (HPLC). Isolation of DEHP required approximately 1 pass through the FC column, 1 through the SEC column, and 4 passes through the HPLC column, per 400 g of cheese. Thus, for a typical batch of cheese averaging 1.6 kg, 4, 4, and 16 column passes were required, respectively.

Liquid Chromatography Separations

Each 2-L acetonitrile sample was split into seven 285-mL portions that were rotary-evaporated and then purified on 7 separate precleaned FC columns using 175 g of 32–63 µm "flash"-grade silica gel particles (Dynamic Adsorbents, Atlanta, GA, USA). The DEHP fractions were typically collected in 300 mL of a 1.6% acetone: 98.4% hexane (volume fraction) mobile phase after elution with 500 mL of 100% hexanes and 1100 mL of the 1.6% acetone solution, as determined by gas chromatography electron-impact-mass spectrometry (GC-EIMS).

Each of the 7 DEHP-containing fractions were subsequently rotary-evaporated to 1 mL and further purified by SEC using tandem 30-cm, 21-mm-ID Oligopore columns (Agilent Technologies, Palo Alto, CA, USA) containing 6-µm particles. DEHP was eluted with 100% methylene chloride and collected in 10-mL fractions. The 7 DEHP fractions were combined, desolvated by rotary evaporation, and reconstituted in 1.5 mL of acetonitrile.

The final purification was achieved with a 15-cm × 9.4-mm-ID C18 column (Agilent Zorbax Eclipse), by eluting with a 95% acetonitrile: 5% water mixture at 4 mL/min for 15 min after elution with 90% acetonitrile: 10% water mobile phase for 20 min. DEHP was observed by UV absorption

at 254 nm and collected in 40-mL borosilicate glass vials with PTFE-lined caps after 33 min. Each sample yielded 80 mL of HPLC eluent from which solvent was removed by rotary evaporation in a 10-mL pear-shaped recovery flask and reconstituted in 1 mL of methylene chloride.

Table 1 Sample and concurrent blank processing.

Process element	Batch 1	Batch 2	Batch 3[a]	Batch 4	Batch 5	Batch 6	Batch 7[b]
Cheese wheel ID	1	1	2	2	2	2	2
Extraction date	8/24/2011	9/26/2011	2/14/2012	2/27/2012	2/27/2012	4/3/2012	4/3/2012
Mass of cheese extracted (g)	2640	2622	1192	1633	1687	1247	1262
Contemporaneously processed blank	1	—	2	3	3	4	4
Number of column passes, isolate/MBlk	35/35	35/na[b]	19/21	23/12	26/12	19/20	21/20

[a]Some of batch isolate lost.
[b]na = blank data not applicable for this batch.

To minimize contamination, glassware, utensils, and aluminum foil coverings were washed, baked at 250 °C for 12 hr, and rinsed with acetone. Sample handling, extraction, and purification methods were performed using glass containers and metallic utensils. LC columns were thoroughly rinsed with the mobile phase that most strongly eluted any stationary phase-retained contaminant DEHP prior to each individual separation.

Analyses

Post-HPLC samples and blanks were analyzed for DEHP and co-eluting compounds by GC-EIMS using an Agilent Technologies (Wilmington, DE, USA) 6890N Network GC system, with a 7683 Series Autosampler, and 5973 *inert* quadrupole mass-selective detector. Analyses were performed with 1.0-µL, on-column injections to a 0.25-mm × 60-m, DB-XLB (Agilent) polysiloxane wall-coated capillary column preceded by a 5-m deactivated fused-silica capillary retention gap, with a 1.3 mL/min helium flow, at an injection temperature of 83 °C. The GC was temperature programmed (hold 60 °C for 3 min; ramp 45 °C/min to 200 °C; followed by 7.5 °C/min to 320 °C; hold 3 min) to elute DEHP at 21 min. The solvent (in each case methylene chloride) was eluted prior to 6 min and, in preliminary analyses, no other compounds could be observed between the solvent elution time and 6 min; and none could be observed between 26 and 76 min after sample injection. Therefore, spectra were collected between 6 and 26 min. Mass scans from 50 to 300 amu (m/z) were acquired every 0.006 min and the entire 251-m/z × 3468-scan matrix was stored for processing. This program and instrument were used for all GC-EIMS analyses to minimize differences in retention times and column-bleed background between subsequent m/z spectra and total ion chromatograms. Three 6-point analytical calibration curves were made with petrogenic DEHP (99.8 ± 0.1% pure; Supelco Analytical, Bellefonte, PA, USA) in each of 2 ranges (70–86 and 0.4–1.5 µg/g in methylene chloride) such that they tightly bracketed the concentration of DEHP in all determinations. Uncertainty of DEHP determinations was determined to be ≤2% in both calibration ranges (M Nelson, unpublished data). Results of these DEHP determinations are listed in Table 2. In addition to DEHP, small amounts of di-n-butyl phthalate, and 28 fatty acids and fatty acid esters were identified in each isolate from its fragmentation ion mass spectra. Each of the latter accounted for 0.04% to 4.10% of the counts in the total ion chromatogram (TIC). Di-n-butyl phthalate accounted for an additional 0.05 ± 0.01% of TIC counts.

Table 2 Analytical results.

Sample ID	Mass C as DEHP[a] (µg)	Purity[b] (%)	Mass C[c] (µg)	GCMS[d] (µg)	$F^{14}C$[e] ($Fm_{reported}$)	$\delta^{13}C$ VPDB[f]
Measured sample carbon						
Isolates						
STO1	92.18 ± 4.65	91.2 ± 2.7	109 ± 3	100.4 ± 5.9	0.284 ± 0.004	–30.0 ± 0.04
STO2	67.25 ± 0.76	88.1 ± 1.7	99 ± 3	76.7 ± 1.5	0.281 ± 0.004	–29.3 ± 0.3
STO3	63.41 ± 0.79	87.2 ± 1.7	127 ± 2	72.5 ± 1.5	0.353 ± 0.003	–29.3 ± 0.3
STO4	65.42 ± 0.76	90.5 ± 1.7	135 ± 2	72.1 ± 1.3	0.311 ± 0.003	–29.3 ± 0.3
STO5	69.09 ± 0.84	92.3 ± 1.4	114 ± 2	75.1 ± 1.3	0.334 ± 0.003	–29.3 ± 0.3
DEHP standards						
STD1	147.5 ± 1	99.8 ± 0.1[g]	135 ± 2		0.0018 ± 0.0042	–29.3 ± 0.16
STD2	143 ± 1	99.8 ± 0.1[g]	130 ± 2		0.0000 ± 0.0044	–29.3 ± 0.16
Whole cheese						
	—	—	—	—	1.045 ± 0.002[g]	–27.1 ± 0.03[g]
Blanks – DEHP "pure" spike and spiked-method blanks						
				Mass C of spike (µg)		
S1	47.32 ± 0.04	99.8 ± 0.1[h]	78 ± 2	47.32 ± 0.04	0.011 ± 0.005	–29.3 ± 0.16
S2	47.98 ± 0.04	99.8 ± 0.1[h]	81 ± 2	47.98 ± 0.04	0.008 ± 0.005	–29.3 ± 0.16
S3	47.75 ± 0.04	99.8 ± 0.1[h]	83 ± 2	47.78 ± 0.04	0.008 ± 0.005	–29.3 ± 0.16
SBlk1	46.05 ± 0.06	ND[i]	68 ± 2	45.39 ± 0.04	0.037 ± 0.005	ND
SBlk2	48.04 ± 0.04	ND	88 ± 2	47.85 ± 0.04	0.022 ± 0.005	ND
SBlk3	47.87 ± 0.04	ND	78 ± 2	45.59 ± 0.04	0.064 ± 0.005	ND

[a]Determined by analytical GCMS analyses, except for gravimetrically prepared aliquots of the Supelco standard DEHP; 1σ combined uncertainty; $n = 3$.

[b]Determined by deconvolution of GC-EIMS data; uncertainty at 95% confidence interval; $n = 5$.

[c]Determined by CO_2 pressure-volume manometry after combustion; 1σ uncertainty; $n = 1$.

[d]Determined by analytical GCMS analyses and purity determinations; 1σ combined uncertainty, $n = 1$.

[e]Modern fraction of carbon in AMS-analyzed sample, corrected for combustion blank of 1 µg C ($Fm_{reported}$); 2σ uncertainty; $n = 1$.

[f]Measured relative to VPDB by gas mass spectrometry. $n = 1$ (ST01), 2 (ST02 through ST05); 3 (STD1 and STD2). Values used for S1, S2, and S3 were that of the mean of STD1 and STD2; 1σ uncertainty.

[g]1σ uncertainty; $n = 3$.

[h]Average and 1σ uncertainty of HPLC-UV and GC-FID determinations; $n = 2$.

[i]ND = not determined.

To permit corrections to be made for isotope fractionation during AMS, chromatographic steps, and biological processes, values of $\delta^{13}C$ with respect to Pee Dee belemnite (VDPB) were determined by $^{13}C/^{12}C$ mass spectrometry (Isoprime, Ltd, Manchester, UK). Three 10- to 15-µg quantities of DEHP isolate from cheese (2 separate samples from wheel 1 and 1 aggregate isolate from wheel 2), three 72- to 100-µg quantities of lyophilized whole Stilton cheese, and three 30- to 61-µg quantities of the Supelco petrogenic DEHP standards were analyzed.

All samples to be analyzed by AMS were reduced to 200 µL in 95% n-hexane and sent from the University of Maryland College Park to LLNL CAMS in borosilicate vials with PTFE-lined caps. These included the 5 DEHP isolates (ST01 through ST05), 3 DEHP-spiked method blanks (SBlk1, SBlk2, and SBlk3), 3 "pure" spikes (S1, S2, and S3), and the ($n = 2$) Supelco petrogenic DEHP standards (STDn) listed in Table 2. Note that both the pure DEHP spikes and standards were submitted without processing except for dilution in hexane and transfer to the shipping vials prior to shipping to LLNL.

At LLNL, samples were transferred to precombusted (900 °C for 3.5 hr) quartz combustion tubes from which solvent was evaporated overnight at 50 °C. Excess CuO was added to each quartz tube. Tubes were evacuated with an oil-free turbopump station, sealed with a H_2/O_2 torch, and heated at 900 °C for 3.5 hr to oxidize all carbon to CO_2. The CO_2 was cryogenically isolated from other combustion products and its carbon mass manometrically determined with a precision of 1.5% to 3.0%, followed by graphitization with iron catalyst in individual reactors (Vogel et al. 1987; Santos et al. 2004). All $^{14}C/^{12}C$ measurements were made on the graphitized samples at LLNL CAMS on the 10MV High Voltage Engineering Europa (HVEE) FN-class tandem electrostatic AMS system. Corrections for background contamination introduced during sample preparation were made following standard procedures (Brown and Southon 1997). All data were normalized to the mean of 6 identically prepared NIST SRM 4990B (oxalic acid I) standards. NIST SRM 4990C, IAEA C-6, and TIRI wood served as quality control secondary standards to monitor spectrometer performance. $^{14}C/C$ concentrations are reported using the $F^{14}C$ (fraction modern) nomenclature for reporting post-bomb data defined in Equation 2 of Reimer et al. (2004). The measurement error was determined for each sample and is reported as 1σ.

Estimation of Isolate Purity

Purity is often determined from the areas of the compounds detected in the chromatogram. To assure that no additional compounds were eluting under the DEHP peak, the DEHP was removed from the GC-EIMS matrix for each isolate by non-negative least-squares deconvolution with that of a Supelco high-purity standard of similar concentration, after reducing the number of time-scans 6-fold by binning to minimize the effects of small retention time variations. Ions detected during the period of DEHP elution were highly correlated and consequently the residual in this region was small (i.e. 0.04% of the counts obtained in the total sample spectrum) and contained no discernible co-eluting peaks. The total residual was free of GC column bleed across the entire chromatogram. The relative masses of co-eluting compounds and DEHP were estimated for each mass fragmentation channel in the residual and isolate spectra. Subsequently, the mass fractions of carbon in the isolate and residual matrices (0.743 ± 0.001 and 0.781 ± 0.002, respectively) were determined as TIC-peak-area-weighted means of the carbon mass fractions of the identified compounds. The purity of carbon from DEHP in each sample was then estimated from the amount of co-eluted carbon determined in the residual, $(N_C)_{residual}$, and isolate matrices, $(N_C)_{isolate}$, i.e.

$$Purity_{DEHP} = 1 - \frac{(N_C)_{residual}}{(N_C)_{isolate}} \quad (1)$$

The calculated purities varied with binning, and consequently their uncertainties were estimated as the standard deviation of results obtained for 5 binning factors (3, 4, 6, 12, and 17). As indicated in Table 2, DEHP purity of the isolates ranged from 88.1 ± 1.4% to 92.3 ± 1.1%.

DATA ANALYSIS

Processing Laboratory Carbon Blanks

In this work, 3 components to the total processing laboratory carbon blank (TBlk) were identified: 1) carbon in the form of DEHP measured in the method blanks; 2) carbon in the co-eluted compounds; and 3) extraneous carbon. They were determined as described below and results are listed in Table 3.

Owing to the differences between batch sizes and collection periods for samples and method blanks, carbon in the 3 method blanks had to be apportioned to represent its contribution to carbon in each

of the 5 isolates. Only DEHP was detected in the method blanks. Its carbon mass contribution to each isolate ($m_{C_{DEHPMBlk}}$) was assumed to scale with the number of LC column passes of the sample relative to that of the blank or blanks to which it was assigned. Since all samples, except for ST01 and ST05, were made by combining portions of isolates from 2 batches and were also associated with 2 different blanks, their contributions from each blank were scaled separately and summed. Isolate ST01 was associated with its own (scaled) method blank value and, for ST05, the method blank value was the scaled average of the DEHP concentration in the 4 method blanks.

The mass of co-eluted carbon, $m_{C_{coel}}$, in each isolate was calculated directly from the isolate's DEHP carbon mass, $(m_{C_{DEHP}})_{isolate}$, and its purity, $(Purity_{DEHP})_{isolate}$. This is

$$m_{C_{coel}} = \left(\frac{m_{C_{DEHP,iso}}}{Purity_{DEHP,iso}}\right)(1 - Purity_{DEHP,iso}) \quad (2)$$

Although DEHP and co-eluted compound carbon blanks could be determined by GC-EIMS, it was evident from differences between manometrically determined C mass (after sample combustion at CAMS; column 4 in Table 2) and our estimates of C mass from analytical GC-EIMS (column 5), that the samples contained substantial amounts of extraneous carbon. This extraneous carbon ($m_{C_{Textr}}$) is attributed to either non-detectable compounds (e.g. polymeric materials) or particles (e.g. detritus, dust, and soot) that deposited in open apparatus (such as flash-chromatography columns and fraction collection containers), or both. The total mass of C in the method blank ($m_{CT_{Blk}}$) is simply the sum of the 3 other components listed in Table 3.

Table 3 Masses of components of total method blank.

Sample ID	$m_{C_{DEHPMBlk}}$ (μg C)[a]	$m_{C_{coel}}$ (μg C)[b]	$m_{C_{Textr}}$ (μg C)[c]	$m_{CT_{Blk}}$ (μg C)[c]
STO1	0.11 ± 0.03	7.47 ± 0.44	8.64 ± 6.14	16.22 ± 6.16
STO2	1.12 ± 0.04	9.49 ± 0.18	22.57 ± 2.69	33.18 ± 2.70
STO3	0.89 ± 0.07	9.05 ± 0.18	54.28 ± 2.58	64.22 ± 2.59
STO4	0.76 ± 0.06	6.67 ± 0.12	62.63 ± 2.49	70.06 ± 2.49
STO5	0.92 ± 0.06	5.97 ± 0.10	38.73 ± 2.49	45.62 ± 2.49

[a] 1σ combined uncertainty; $n = 3$.

[b] 1σ combined uncertainty; $n = 1$.

[c] Total laboratory carbon blank calculated as the sum of the method, co-eluted, and total extraneous blank values listed; 1σ combined uncertainty; $n = 1$.

Laboratory Blank Corrections to *Fm*

The values of $F^{14}C$, denoted as $Fm_{reported}$ in the following calculations, are listed in Table 2 and are linear combinations of the modern fraction of the DEHP carbon in each isolate ($Fm_{DEHP,iso}$), and that of the total laboratory carbon blank (Fm_{TBlk}). Thus,

$$Fm_{reported} = Fm_{DEHP,iso} \cdot \gamma_{DEHP} + Fm_{Tblk} \cdot \gamma_{Tblk} \quad (3)$$

where γ_{DEHP} and γ_{TBlk} are the mass mixing ratios of the DEHP carbon in the isolate and the total laboratory blank, respectively. Noting that $\gamma_{DEHP} = (1 - \gamma_{TBlk})$, the laboratory blank-corrected values of the modern carbon fractions of the DEHP (Fm') in the isolates were calculated from data in Tables 2 and 3 as follows:

$$Fm_{isolate}' = \frac{Fm_{reported} - Fm_{TBlk} \cdot \gamma_{TBlk}}{1 - \gamma_{TBlk}} \quad (4)$$

where

$$\gamma_{TBlk} = \frac{m_{CT_{Blk}}}{m_{CT_{Blk}} + m_{C_{DEHP,iso}}} \quad (4.1)$$

In Equation 4, Fm_{TBlk} for each isolate was calculated from the linear combination of its 3 laboratory blank components, i.e.

$$Fm_{TBlk} = \gamma_{DEHP_{MBlk}} \cdot Fm_{DEHP_{MBlk}} + \gamma_{Coel} \cdot Fm_{Coel} + \gamma_{extr} \cdot Fm_{extr} \quad (5)$$

where γs are mass mixing coefficients defined as follows:

$$\gamma_{DEHP_{MBlk}} = \frac{m_{C_{DEHPMBlk}}}{m_{C_{TBlk}}}, \gamma_{DEHP_{MBlk}} = \frac{m_{C_{Coel}}}{m_{C_{TBlk}}}, \text{ and } \gamma_{extr} = \frac{m_{C_{extr}}}{m_{C_{TBlk}}} \quad (5.1), (5.2), (5.3)$$

each of which was calculated with data in Table 3. Method blanks were deemed to contain too little mass for AMS and so the values of $Fm_{DEHP_{MBlk}}$ were assumed to be the mean of values (0.0009) reported for the petrogenic Supelco DEHP standard (Table 2). As the co-eluted mass consisted of natural fatty acids and fatty acid esters from the cheese matrix, Fm_{Coel} was assigned to that of the lyophilized whole cheese (Fm = 1.045 ± 0.002). The modern fraction of extraneous carbon in Equation 5, Fm_{extr}, was determined from the spiked-blank data.

Determination of *Fm* values for Extraneous Carbon

As can be determined from Table 2, the *Fm* values reported for the pure spikes (0.009 ± 0.001, average and standard error of the mean for pure spikes S1, S2, and S3) were uniformly 10-fold greater than those (0.0009 ± 0.003) reported for the much larger DEHP standard. Thus, it was clear that the "pure" spikes contained some extraneous modern carbon mass and that it was accrued during post-GC-EIMS operations (dilution, packaging, and shipping). Herein, this post-GC-EIMS component of the extraneous carbon mass ($m_{mC_{extrPostGCMS}}$), was readily calculated from the measured value of the *Fm* reported for each "pure" spike $Fm_{reported_{spikeS}}$) and its manometrically determined carbon mass ($m_{C_{CO_2,spikeS}}$) as follows:

$$m_{mC_{extrPostGCMS}} = m_{C_{CO_2,spikeS}} \cdot Fm_{reported_{spikeS}} - m_{C_{spikeSDEHP}} \cdot Fm_{spikeS} \quad (5.4)$$

where the product $m_{C_{spikeSDEHP}} \cdot Fm_{spikeS}$ is the mass of modern carbon in the DEHP used to prepare each "pure" spike (S1, S2, or S3) and Fm_{spikeS} is again, that of the Supelco standard, i.e. 0.0009 ± 0.0009. The average $m_{mC_{extrPostGCMS}}$ was 0.725 ± 0.12 μg C and was assumed to be applicable to all isolates, except ST01.

The remaining modern extraneous carbon is clearly attributed to pre-GC-MS processing (i.e. $m_{mC_{extrPreGCMS}}$) and was determined analogously for each of the spiked method blanks from its mass ($m_{mC_{CO_2SBlk}}$) and measured modern fraction ($Fm_{reported_{SBlk}}$) as follows:

$$m_{mC_{extrPreGCMS}} = m_{mC_{CO_2SBlk}} \cdot Fm_{reported_{SBlk}} - m_{C_{SBlkDEHP}} \cdot 0.0009 - 0.725 \ \mu g \quad (5.5)$$

Values of $m_{mC_{extrPreGCMS}}$ calculated in this way were apportioned to each respective isolate as outlined above for the apportioning of $m_{C_{DEHPMBlk}}$. The apportioned values are listed in Table 3. The total extraneous modern carbon masses, and pre- and post-GCMS attributed to each isolate ($m_{mC_{Textrisolate}}$) are listed in Table 4. Finally, the values of Fm_{extr} needed to calculate Fm_{TBlk} for each isolate using Equation 5 were determined using the definition

$$Fm_{extr} = \frac{m_{mC_{Textrisolate}}}{m_{C_{Textr}}} \tag{5.6}$$

and these are also listed in Table 4.

Table 4 Reported and component blank fractions of modern carbon values used to determine $Fc_{DEHP_{cheese}}$ of each isolate.

Sample ID	$Fm_{reported,iso}$[a]	Fm_{extr}[b]	Fm_{TBlk}[c]	$Fc_{DEHP_{cheese}}$[d]
ST01	0.284 ± 0.004	0.190 ± 0.090	0.580 ± 0.497	0.213 ± 0.032
ST02	0.281 ± 0.004	0.435 ± 0.068	0.593 ± 0.117	0.128 ± 0.040
ST03	0.353 ± 0.003	0.220 ± 0.023	0.333 ± 0.039	0.302 ± 0.044
ST04	0.311 ± 0.003	0.208 ± 0.012	0.285 ± 0.029	0.277 ± 0.042
ST05	0.334 ± 0.003	0.255 ± 0.098	0.353 ± 0.137	0.253 ± 0.037

[a] 2σ uncertainty; $n = 1$.
[b] 1σ combined uncertainty; $n = 1$.
[c] 1σ combined uncertainty; $n = 1$.
[d] 1σ Monte Carlo-derived uncertainty; $n = 100{,}000$.

Fractionation Corrections and Contemporary Carbon Calculation

For the purpose of this study, the *Fm* values reported in Table 2 were converted to their corresponding fractions of "contemporary" carbon, *FC,* determined with respect to the value of *Fm* of the lyophilized whole cheese matrix and corrected for ^{14}C fractionation as per Stuiver and Polach (1977) as shown in Equation 6. Use of whole cheese as a contemporary reference standard eliminates the need to correct for differences in ^{14}C fractionation between the cheese and the oxalic acid standard, as well as for fluctuations in atmospheric ^{14}C since 1950 due to nuclear weapons testing.

$$FC_{DEHP_{cheese}} = \left(\frac{Fm_{isolate}'}{Fm_{wholecheese}}\right)\left[\frac{\left(1-2\frac{(\delta^{13}C_{VPDB})_{isolate}}{1000}\right)}{\left(1-2\frac{(\delta^{13}C_{VPDB})_{wholecheese}}{1000}\right)}\right] \tag{6}$$

In this equation, $FC_{DEHP_{cheese}}$ is the fraction of contemporary carbon from DEHP in the isolate after all corrections, and thus that in the cheese sample, and $Fm_{wholecheese}$ is the fraction of modern carbon reported for the lyophilized whole Stilton cheese (1.04 ± 0.008). Respective $\delta^{13}C$ (VPDB) are listed in Table 2. The fraction of contemporary carbon in isolates from Stilton cheese, $Fm_{reported,iso}$, along with their respective Fm_{extr}, Fm_{TBlk}, and calculated fractions of contemporary carbon, *FC*, of DEHP are presented in Table 4.

Uncertainty Analysis

Uncertainties in $FC_{DEHP_{cheese}}$ values reported herein (Table 4) were estimated using the Monte Carlo method as follows. Equation 6 and supporting Equations (3–5) for calculation of derived blank masses, blank mixing ratios, and modern fractions were coded into a Matlab® script for the calculation of $FC_{DEHP_{cheese}}$ of each isolate. Specifically, blank carbon masses ($m_{C_{coel}}$, $m_{C_{Textr}}$, $m_{C_{TBlk}}$, $m_{mC_{extrPostGCMS}}$, $m_{mC_{extrPreGCMS}}$), mass mixing ratios (γ_{TBlk}, $\gamma_{DEHP_{MBlk}}$, $\gamma_{DEHP_{MBlk}}$, and γ_{extr}), and blank modern fractions (Fm_{TBlk} and Fm_{extr}) were calculated as per the equations and relationships described above. Values of $m_{C_{DEHPMBlk}}$, $m_{C_{DEHP,iso}}$, PVM carbon mass, isolate purity, the remaining modern fractions (Fm_{Coel}, $Fm_{DEHP_{MBlk}}$, $Fm_{reported}$, and $Fm_{wholecheese}$), and $\delta^{13}C_{VPDB}$ were input as received from the measurement laboratories. We note that $Fm_{reported}$, CO_2 manometrically derived (PVM) carbon masses, and $\delta^{13}C_{VPDB}$ were input for all sample IDs listed in Table 2, i.e. including those for the spikes and spiked blanks. These input variables, totaling 16, are listed in Table 5 for isolates, spikes, and spiked blanks. Subsequently, for each of the 16 variables, the Matlab function, *randn*, was used to populate a matrix of 100,000 inputs randomly chosen from a normal distribution of values centered at the mean of each variable and having a standard deviation corresponding to the uncertainty in each. Uncertainties in all entered variables were estimates of 1σ, except for Fm_{DEHP} and $Fm_{reported}$ where 2σ uncertainties were used. In the latter, individual measurement uncertainty exceeded the standard deviation estimate of the 2 values and so a 2σ uncertainty of the mean was used.

Table 5 Example input parameters used with Monte Carlo analysis determination of $FC_{DEHP_{cheese}}$ and uncertainty assessment.

Parameter	Description of parameter	Sample ST05	SBlk1
PVM	Mass of C by $C0_2$	114 ± 2	68 ± 2
$m_{C_{DEHP,iso}}$	Mass of C as isolated DEHP	69.09 ± 0.84	0.68 ± 0.05
$m_{C_{spikeSDEHP}}$	Mass of C in DEHP spike	—	45.39 ± 0.04
$m_{C_{DEHPMBlk}}$	Mass C DEHP in apport. blank	0.92 ± 0.06	—
Fm_{Coel}	Modern fraction co-eluted	1.045 ± 0.002	—
Fm_{DEHP}	Modern fraction fossil DEHP	0.0009 ± 0.009	0.0009 ± 0.009
Purity	By deconvolution	0.918 ± 0.014	—
Fm_{reported}	Fraction modern of sample	0.3335 ± 0.0031	0.037 ± 0.005
$Fm_{wholecheese}$	Fraction modern of whole cheese	1.0405 ± 0.0081	—
$(\delta^{13}C_{VPDB})_{isolate}$	δ^{13}C fractionation for the isolate	–29.3 ± 0.3	—
$(\delta^{13}C_{VPDB})_{wholecheese}$	δ^{13}C fractionation for the whole cheese	–27.1 ± 0.03	—
$(\delta^{13}C_{VPDB})_{DEHP}$	δ^{13}C fractionation for the DEHP	—	–29.1 ± 0.04

RESULTS AND DISCUSSION

As indicated in Table 4, *FC* of DEHP from cheese ranged from a low of 0.128 ± 0.040 (ST02) to 0.302 ± 0.044 (ST03) and the mean of Monte Carlo-perturbed values calculated for all isolates was 0.235 ± 0.073 (1σ, and ± 0.091 at 95% confidence interval), indicating that most of the DEHP was petrogenic. The value for ST02 appears inordinately low. However, applying the Grubbs (1969) test, we find that its difference from the Monte Carlo mean is not significant at the 95% confidence level and, thus, is not a statistical outlier. This value could be the result of an inhomogeneity of DEHP in the cheese. Not surprisingly, we find from sensitivity calculations, that *FC* values are sensitive to their corresponding $m_{C_{Textr}}$ values, and consequently increase with increasing carbon mass values

determined from CO_2 measurements after combustion, from which the latter are calculated. If the manometrically derived value for ST02 were 139.4 µg, i.e. representative of a 63-µg extraneous carbon mass (and more comparable to spiked blank SBlk1), instead of 22.3 µg, then its *FC* would be 0.291 ± 0.042. Moreover, we note that *Fm* values are independent of the carbon mass (so long as the mass is homogenous) and that there was little difference in the $F^{14}C$ values reported for ST02 (0.281 ± 0.004) and the mean (0.333 ± 0.021) for ST03, ST04, and ST05. We suspect, therefore, that the entire sample was not combusted to CO_2, possibly owing to loss during transport. The difference between 139.4 µg and the manometrically derived value of 99 µg used in the analysis above is equivalent to the loss of 50 µL of the sample in solution prior to combustion.

As noted above, the total carbon blank, averaging 45 µg, was determined to be substantial. About 17% of this was co-eluted carbon derived from the cheese matrix and therefore expected. The remaining amount represents extraneous carbon, of which from analysis of the pure spikes, 30 µg (i.e. ~80%), could only be associated with the shipping vials. As the caps could not be baked, they are likely the source of this contamination. In subsequent tests, hexadecamethylheptasiloxane, a component of the silicone septum affixed to the top of the Teflon™ liner, could be readily detected by GC-EIMS after pure hexane was shaken in a clean vial that had been successively uncapped and recapped as required to switch from the acetonitrile-water HPLC eluent to hexane and to perform concomitant rotary evaporations. It is notable that this compound was not detectable when the vial was capped and reopened only once. However, the vial caps had been washed with a dilute solution of Alconox™, for which a preliminary $Fm_{reported}$ was subsequently determined by AMS to be 0.28 ± 0.03, i.e. comparable to our estimates of Fm_{extr} values listed in Table 4. Thus, it appears that Alconox residue was largely responsible for the extraneous carbon mass in our isolates.

CONCLUSIONS

We have utilized small-scale CSRA by AMS to determine the biogenic fraction of a food contaminant in a fatty food matrix at concentrations well below 1 mg/kg. The purity of the isolates prepared for AMS analysis was assessed, as well as the influence of extraneous contaminant carbon from all steps of the sample preparation and analysis. Our results indicate that the majority of DEHP extracted from the 2 Stilton cheese wheels is of an industrial, petrogenic nature. However, consistently higher-than-fossil values of corrected *FC* indicate that a significant portion of DEHP present in the cheese is of modern, likely biogenic, origin. This is in accordance with and strongly supportive of previous studies suggesting that bis(2-ethylhexyl) phthalate is naturally produced by certain algae, mold, and, likely in this case, strains of penicillium.

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