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ARTICLE

Multiresidue Method for Pesticides and Persistent Organic Pollutants (POPs) in Milk and Cream Using Comprehensive Two-Dimensional Capillary Gas Chromatography−Time-of-Flight Mass Spectrometry

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A method for the analysis of pesticides and their metabolites including most of the persistent organic pollutants (POPs) in milk and cream is described. The method was single-laboratory validated through milk fortification in quadruplicate with 34 pesticides, isomers, and metabolites including 12 of the insecticide POPs and their metabolites. Whole cow's milk was fortified at 0.2, 0.4, 1, 2, 10, or 50 μg/kg wet weight and extracted with acetone/cyclohexane/ethyl acetate (2:1:1) with the addition of Mg_2SO_4 and NaCl. Fat recovered in the extract accurately reflected the fat content of the milk or cream. All test portions were purified on a gel permeation chromatograph (GPC) followed by solid phase extraction (SPE) cleanup on a mixed bed graphitized carbon black (GCB) and primary/secondary amine silica gel (PSA) column before determination using a comprehensive two-dimensional gas chromatograph interfaced to a time-of-flight mass spectrometer. Average recoveries were 77, 72, 73, 66, 77, and 84% for 0.2, 0.4, 1, 2, 10, and 50 μg/kg wet weight whole milk, respectively. The average relative standard deviations for 0.2, 0.4, 1, 2, 10, and 50 μg/kg were 10, 8, 7, 7, 3, and 3%, respectively. The limits of quantification (LOQs) for all pesticides were 0.2 or 0.4 μg/kg wet weight. An archived cream sample collected in 1982 on Oahu, Hawaii, was found to contain only hepatachlor epoxide (HE) and DDE-*p,p′* at 380 ± 24 and 69 ± 17 μg/kg fat, significantly elevated over the current action level of 50 μg/kg fat for HE.

KEYWORDS: Multiresidue methods; fat determination; persistent organic pollutant; pesticides; gel permeation chromatography; SPE; GC×GC-TOFMS; two-dimensional GC

INTRODUCTION

The determination of pesticides in foods with high fat content presents difficulties to analytical methods. The pesticides under investigation in this study are found associated with the fatty portion of food. This is especially true for the persistent pesticides in animal- and vegetable-derived fats. Because fats are recovered in the extraction steps, these fats must be removed before measurement by GC-MS techniques.

A large number of physical and chemical approaches have been proposed to remove coextracted fats including liquid/solid extraction, (*1*−*6*, *13*), liquid/liquid partitioning with polar solvents (*6*, *13*) such as acetonitrile (MeCN), or freezing out of the fat (*10*). Supercritical fluid extraction requires the addition of modifying solvents (*12*). Other methods use absorption on solid phase extraction columns and matrix solid phase dispersion (MSPD) using C_{18} (*11*), alumina (*10*), or Florisil (*4*, *5*) or MSPD using C_8, C_{18} (*18*), Celite (*2*), or amino propyl silca gel (*7*, *14*, *20*) or alumina followed by column extraction with normal phase column chromatography (*8*).

Gel permeation chromatography has often been selected for separating larger amounts of lipids from smaller molecules of interest (*1*, *9*, *13*, *19*, *26*). Lipid removal requires large quantities of toxic solvents such as dichloromethane (DCM) and sorbents or columns for ∼1−4 g of fat contained in a test portion. Liquid/liquid extraction (LLE) techniques using MeCN and hexane, DCM, or other solvents are tedious and can sometimes produce lower recoveries for pesticides that are the most persistent and very lipid soluble such as the pesticide POPs. LLE has often been used prior to GPC or MSPD techniques as well (*1*−*3*, *6*, *7*, *11*, *13*). The difficulty associated with the analysis of fatty foods for pesticides has been recently reviewed by Gilbert-Lopez et al. (*13*). A large number of procedures have been proposed for high-fat animal- and plant-derived foods (*13*). Gilbert-Lopez et al. suggested that sample preparation was critical to the analysis of pesticides even with new hyphenated mass spectrometry techniques. Some approaches reduce the sample size and/or dilute or at least not concentrate the extract to achieve a sufficiently clean extract for GC or GC-MS (*13*).

The U.S. Food and Drug Administration is responsible for the enforcement of pesticide tolerances in foods for currently registered pesticides and also for enforcing action levels of pesticides no longer registered that continue to be found in foods for which

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tolerances have been revoked. These action levels are generally expressed in lipid-adjusted values for milk such as for heptachlor epoxide (HE), 50 μg/kg milk fat or 1.6 μg/kg wet weight, and for cream, 18.5 μg/kg wet weight. The 1,2,3,4,5,6-hexachlorocyclohexane (BHC) tolerance is 300 μg/kg fat for total BHCs (α, β, γ, δ, ε) or 60 μg/kg fat each, assuming an equal distribution of the isomers or ~2 μg/kg wet weight whole milk and ~20 μg/kg wet weight for cream. The fat content of foods must therefore be known to express the results correctly.

We investigated some methods of fat determination as part of an overall analytical method. Our primary concern in method development for milk was to achieve a method with LOQs sufficiently lower than the action levels and tolerances to ensure accurate measurement at the action level or tolerance. The method needs to be "fit for purpose" as has been discussed previously (*15*). LOQs at least 5 times lower than the tolerance and/or action level should be sufficient (*16*). Method validation should be done at the stated LOQs to demonstrate method effectiveness followed by the analysis of incurred milk samples or a certified reference material, if available. Some methods have not demonstrated their effectiveness in a concentration range low enough for U.S. FDA action levels in milk or cream (*2*, *4*−*6*), whereas some others have (*9*, *19*).

Recently, pesticide extraction and cleanup of fruits and vegetables has been streamlined by use of the QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method, which uses smaller sample sizes, an excess of salts, and MeCN to remove pesticides from fruit and vegetable matrices (*14*) and has been modified for higher fat foods such as egg, milk, avocado, and olive oil (*5*, *6*). After extraction, a portion of the MeCN extract is cleaned up by dispersion with primary and secondary amine silica (PSA) with more drying agents. The procedure has distinct advantages of being very fast using minimal material and sample handling and, as originally described, no concentration of the sample is typically used (*5*, *6*). Using the QuEChERS procedure, fat coextraction is relatively low as is the recovery of POPs from higher fat matrices (*5*, *6*).

Sannino et al. (*18*) modified a procedure based on that of Specht et al. (*9*) which involved acetone extraction of pesticides in fresh produce samples, followed by a partitioning step involving a 1:1 (v/v) mixture of ethyl acetate (EtOAC)/cyclohexane. In our previous work with the dietary supplement ginseng, we developed a procedure that uses GPC and SPE cleanup that proved to be effective on a number of dietary supplement herbs and root powders for 170 pesticides (*17*). In this paper, we combine a modified extraction by Sannino et al. (*18*) with the effectiveness of a dietary supplement cleanup for use on milk and cream. We selected all of the pesticide POPs for study except toxaphene, which is a large mixture of isomers and chlordecone. In addition, some other organochlorine pesticides and metabolites found in milk along with a few more organophosphorous and organochlorine pesticides were selected to give more variety. Results using this procedure are presented for the analysis of milk fortified with 34 pesticides, over a 250-fold concentration range bracketing the pesticide action levels in milk. HE and DDE levels are also determined in an incurred cream embargoed during a HE incident during 1982 in Hawaii.

MATERIALS AND METHODS

Materials and Standards Preparation. The majority of pesticide standards were obtained from the U.S. Environmental Protection Agency (U.S. EPA) National Pesticide Standard Repository (Ft. Meade, MD). Other pesticides were purchased from Sigma-Aldrich (Fluka, Milwaukee, WI), and Chem Service Inc. (West Chester, PA). Pesticide-grade ethyl acetate (EtOAC), hexane, ethanol, diethyl ether (DEE), dichloromethane (DCM), methanol, pentane, acetonitrile (MeCN), cyclohexane, acetone, and toluene, HPLC-grade water, and certified-grade anhydrous sodium sulfate, anhydrous magnesium sulfate, and sodium chloride were purchased from Fisher Scientific (Pittsburgh, PA). The internal standards, acenaphthene-d_{10}, phenanthrene-d_{10}, and chrysene-d_{12}, were purchased from Sigma-Aldrich. Combined primary and secondary amine and graphitized carbon black 6 mL solid phase extraction columns were purchased from United Chemical Technologies (Bristol, PA). Whole pasteurized milk and cream were purchased in College Park, MD, for fortification and fat extraction studies. An archival cream sample kept frozen since 1982 was generously provided by the Hawaii Heptachlor Research and Education Foundation (HHR&EF). All samples and extracts were kept at −40 or −20 °C, respectively.

Table 1. Pressurize Solvent Extraction Relative Fat Recoveries Using ASE 300 with Liquid/Liquid Extraction Using Diethyl Ether/Hexane Set to 100%[a]

PSE method	% of LLE	*n*
acetone/Cyc-Hex (2:1)	71	1
acetone/DCM/Cyc-Hex (4:3/:3)	66	1
EtOH/toluene (7:3)	81	1
MeOH/DCM (1:4)	72	1
MeOH/DCM (1:2)	90	1
DCM/Hex (1:1)	80	1
EtOH/DCM/Hex (1:2:2)	71	2
MeOH/DCM/pentane (1:2:2)	97	1
MeOH/DCM/Hex (1:2:5)	81	5
MeOH/DCM/Hex (1:4.5:4.5)	100	1
MeOH/DCM/Hex (1:2:2)	104	4

[a] ASE conditions: 1500 psi, 80 °C, 5 min static extraction, 60% flush, 100 s purge, two cycles.

Stock solutions of individual pesticide standards were prepared by dissolving between 52.0 and 65.7 mg of each pesticide in 25 mL of toluene for fortification and calibration standards. The working standards mixture used for calibration and fortification was prepared by mixing between 0.758 and 0.962 mL of each standard in a 100 mL volumetric flask sufficient to give a 20.0 mg/L working standard concentration for each pesticide. The lower fortification solutions were prepared in toluene by dilution of the 20 mg/L working standard to 2.0 and 0.2 mg/L prepared in toluene for calibration or 1.0 and 0.1 mg/L in acetone for fortification. Dilution of the 2.0 or 0.2 mg/L stock pesticide standards in toluene were used to prepare 1, 2, 5, 10, 25, 50, 100, 200, 500, and 1000 μg/L standards in toluene for GC×GC-TOFMS optimizing prior to most fortifications. Matrix-matched standards were prepared for quantifying fortifications and incurred samples by adding stock pesticide standard mixtures at 0.2 and 2 mg/L to blank milk extracts prepared alongside each fortification using 20 g milk aliquots to give 1, 2, 5, 10, 25, 50, 100, and 200 μg/L. The internal standards were prepared by dissolving working solutions in toluene of deuterated polycyclic hydrocarbons to a 2.5 mg/L working solution in toluene. Internal standards at 100 μg/L were also added to the matrix-matched standards. Calibration curves were constructed using the matrix-matched standards and a single quantitation ion from each pesticide or POP.

Fat Determinations. Fat was determined gravimetrically in milk and cream on a Mettler electronic analytical balance (model AE 240). Milk or cream fats were extracted as described below. The entire extract of milk or cream was evaporated on a weighed aluminum pan to constant weight, except the Hawaii cream, for which a 4 mL aliquot was used. Alternately, fat was extracted by LLE of cow's milk and cream performed by an AOAC fat extraction method (*26*). The ratio of solvents, ethanol/DEE/hexane, was 1:1:1 (v/v/v), and potassium oxalate was added (*26*). The collected organic layer was filtered and dried by anhydrous sodium sulfate Na_2SO_4 into a 1 L round-bottomed flask. The extract was evaporated by a rotary evaporator and transferred to an aluminum weigh boat to determine the lipid content gravimetrically. Whole milk was also freeze-dried for pressurized solvent extraction (PSE) of the fat using 11 test solvent systems provided in **Table 1**. The milk sample aliquots were frozen at −39 °C and freeze-dried for 30 h at −40 °C and at 300 × 10^{-3} mbar. A 6 g portion of the freeze-dried milk sample was ground finely with Na_2SO_4 and was filled into a 100 mL cell for an ASE 300 (Accelerated Solvent Extractor "ASE" Dionex, Sunnyvale, CA) as illustrated in **Figure 1**. The ASE parameters

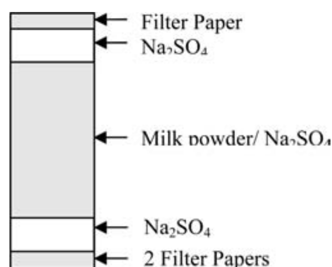

Figure 1. Cell packing for 100 mL cell of the ASE 300.

used for the method are listed in **Table 1**. During evaporation of the extracts, a small amount of DCM was added to prevent complete evaporation of methanol. The lipid extract was dried in an aluminum weigh boat to determine the lipids gravimetrically.

Milk and Cream Procedure. Milk was thawed and remixed, and 20 g portions were used for test fortifications. The Hawaiian heavy cream was thawed, and 1.5 g portions of the solids were weighed in triplicate. Each portion was mixed with 18.5 g of HPLC-grade water and vortexed vigorously. Milk portions weighed into 50 mL centrifuge bottles were fortified in quadruplicate with either 40, 80, or 200 μL of a 0.1 mg/L pesticide mixture or 40, 200, or 1000 μL aliquots of a 1.0 mg/L stock pesticide standard mixture in acetone. These fortifications produced concentrations of either 0.2, 0.4, 1.0, 2.0, 10.0, or 50.0 μg/kg wet weight in the whole milk. The standards were allowed to mix with the milk for 1 min. Twenty milliliters of a mixture of acetone/EtAOC/cyclohexane (2:1:1) was added. Eight grams of Mg_2SO_4 plus 1.5 g of NaCl was then added. The mixture was shaken for 1 min at 1000 strokes/min on a Geno-grinder (Supex Corp.). The mixture was centrifuged for 5 min at 4500 rpm (4200*g*) (ThermoElectron Corp., Milford, MA). The supernatant consisted of 15−16 mL of clear solvent for milk and was removed for cleanup and fat analysis.

Gel Permeation Chromatography (GPC). The entire 15−16 mL extract from milk or 8 mL for cream was reduced to <3 mL in EtOAC/cyclohexane and dried of the traces of remaining water using magnesium sulfate while being filtered through a 0.2 μm Teflon filter and made up to 5 mL in GPC mobile phase. The 5 mL extracts were applied to an express GPC column using the autosampler and HPLC pump in an Accuprep automated GPC system (J2 Scientific, Columbia, MO) The express columns consisted of 24 g S-X3 Bio-beads swelled in 50:50 EtOAC/cyclohexane. The flow rate was 5 mL/min with a 22 min cycle time. Fat was discarded during the initial 0−10 min of each GPC cleanup, whereas pesticides were collected in the following 10−20.5 min.

SPE Column Cleanup. Pesticide-containing fractions were reduced to approximately 1 mL on a Labconco Rapidvap vacuum evaporator at 45 °C and 240 Torr for ~20 min. A set of SPE columns was prewashed with 5 mL of 50:50 EtOAC/cyclohexane. The extract in EtOAC/cyclohexane was applied to a PSA (500 mg)/GCB (250 mg) SPE column and eluted with 13 mL of 75% acetone/toluene. The entire extract was reduced to 1 mL under nitrogen gas and transferred to a vial containing 100 ng of acenaphthene-d_{10}, phenanthrene-d_{10}, and chrysene-d_{12} as internal standards.

GC×GC-TOFMS Analysis. All milk and cream extracts were measured for pesticide content using a Pegasus 4D GC×GC-TOFMS (LECO Corp. USA). The Pegasus 4D was equipped with a 7890N Agilent gas chromatograph containing a deactivated guard column (5 m × 0.25 mm i.d., Agilent Technologies, Little Falls, DE) connected to a 30 m × 0.25 mm i.d., 0.25 μm film, VF-5 ms (Varian Corp.) for the first dimension and a 2.2 m × 0.1 mm i.d. BPX-50 (SGE Corp.) (*22*) or alternately a non-silicone-arylene phase, HP5 ms, 30 m × 0.25 mm i.d., 0.25 μm film (Agilent Technologies), with a 1.5 m × 0.15 mm i.d., 0.15 μm film, BPX-50 (SGE Corp.) selected on the basis of the results reported by Beens et al. (*21*). The column phases selected are commercially available and known to provide reasonable pesticide or POP separations (*22*, *23*). Helium was the carrier gas. We used a constant pressure of 55 psig (379 kPa) or 29 psig (200 kPa) with the 1.5 m secondary column (*21*, *22*). The constant pressure was used to maintain more optimal flow in the primary column at the beginning of the GC temperature program and a more optimal flow through the secondary column toward the end of the GC temperature program (*21*, *22*). The temperature program was 90 °C for 2 min, then 10 °C/min to 180 °C, and then 4 °C/min to 290 °C. The second-dimension column was kept at a constant 35 °C offset for the 2.2 m × 0.1 mm i.d. BPX-50 column and at a 50 °C offset with the 1.5 m × 0.15 mm i.d. BPX-50 second-dimension column. These offsets were used to help reduce wraparound with a 3 Hz modulation for higher boiling strongly retained or aromatic compounds such as chrysene, mirex, PCBs, or even PBDEs, if present in real milk samples (*37*). The total time to elute the latest eluting POP investigated, mirex, was 37.6 min (2257 s), using the longer and narrower secondary column, or 26.6 min (1596 s), using the shorter and wider secondary column. The modulator offset was 50 °C (65 °C in the second-column set) relative to the primary oven (*24*). The ion source and interface temperatures were set to 230 and 280 °C, respectively (*22*). The modulation period was 3 Hz, hot pulse 0.7 s, and cold pulse 0.8 s (*24*). The spectral acquisition rate was set to 200 Hz recorded between m/z 40 and 600, and the MCP detector was set at 1750 V (*22*).

Five microliters of extract was injected for all samples and matrix-matched standards, except the initial 50 ppb test spike (1 μL and for matrix standards as well). The matrix-matched standards and milk extracts were injected into a CIS4 injector with a baffled glass insert at 0.8 μL/s using an MPS2 autosampler with a 10 μL syringe (Grestel Corp.) (*22*, *25*). The temperature was set initially at 40 °C while solvent venting at 200 mL/min for 18 s (*22*, *25*) including the time to inject the sample. The CIS4 was programmed at 10 °C/s to 280 °C. All samples and matrix-matched standard were deconvoluted using LECO Chromatof 4.13 version software. Deconvoluted spectra were searched against both a user-created library (pesticide1) and the NIST05 library. The user-defined library contained the spectra for 155 pesticides. Minimum similarity for naming compounds was set to 600 and 500 for combing slices. A linear calibration curve from the matrix-matched standards acquired on the same day was used for each pesticide to calculate recoveries.

Statistics, Analysis, QC, and Calculations. Milk matrix blanks were analyzed with all milk and cream determinations. All fortifications were analyzed in quadruplicate, and all incurred samples were analyzed in triplicate. Chromatof version 4.13, deconvolution software, was used to identify pesticides. Pesticides found in fortified or incurred samples were required to match the retention times in both column dimensions to within one modulation in the first dimension and ±0.02 s (or ~±1%) in the second dimension for the matrix-matched standards prepared during that analysis. The retention time for the first dimension is assigned by the Chromatof software to the slice that is the largest, the "base peak". We allowed the base peak to shift earlier or later by one slice without flagging peak with an error for the retention time of the pesticide. The similarity to the library spectrum had to be >550, and the spectrum must match the calibration reference spectrum generated from the matrix-matched standards (**Table 3**). Pesticide concentrations were calculated from a linear standard curve from matrix-matched standards prepared on the same day. All means and standard deviations were calculated in a Microsoft Excel spreadsheet after copying the results calculated in Chromatof from each sample's peak table. HE was also calculated in the Hawaiian cream sample by method of additions. Five standard addition solutions were prepared using a 200 μL aliquot of the final extract diluted 1:1 with only toluene, 50 ng/mL, or 100, 150, and 200 ng/mL HE standard in toluene. All five dilutions were analyzed by injecting 1 μL into the GC×GC-TOFMS, and the area for m/z 353 was plotted against concentration.

RESULTS AND DISCUSSION

Fat Extractions and Fat Determinations. Whole milk fat content was determined using liquid/liquid extraction or liquid solid extraction with pressurized solvents of the solids remaining after freeze-drying of the whole milk. Eleven solvent systems were tested with freeze-dried milk to determine lipid content, and two liquid/liquid solvent systems were tested with fresh whole milk or heavy cream. Freeze-dried milk equivalent to 100 g wet weight was placed in a 100 mL ASE cell as exhibited in **Figure 1**. Each dried milk sample was given two 5 min static extractions. The PSE conditions used were identical with each solvent system, and the results obtained are listed in **Table 1**. Two solvent systems using specific ratios of MeOH, DCM, and hexane or pentane were

Table 2. Fat Determinations in Heavy Cream Using a Single Solvent Extraction (15 mL) and a Salting-out Step Compared with an AOAC Repetitive Liquid/Liquid Extraction Method

trial	salt-out 2:1:1 recovery % of 15 mL	salt-out % fat in cream assumes 15 mL	% fat recovery in extract assumes 37.0% = 100	LLE PAM (*1*) 304 E4% fat	USDA database (*34*) cream
1	95	37.7	97	36.4	37.0
2	95	37.1	95	36.3	37.0
3	95	36.5	94		37.0
4	93	36.2	91		37.0
mean	95	36.9	94	36.4	37.0

more efficient than other systems previously reported when compared with the LLE procedure (*27−32*). The two most efficient PSE extractions using the solvents MeOH/DCM/Hex did not yield different results from the LLE method of AOAC (*26*). This AOAC LLE procedure was used as a reference for the second LLE procedure that used a modified procedure reported by Sannino et al. (*18*). Both milk and heavy cream test portions were subsequently extracted with both procedures as described previously. The AOAC LLE is a more tedious procedure, but avoids a drying step required with liquid/solid extraction. This procedure can be made more efficient by reducing the number of extractions to one and decreasing the sample size while reducing the solvent volume. If this is done, then the desired LOQs using the same detection system will need to be maintained by either more concentration or larger injection volumes or both.

The procedure used for analyses in this study uses a single extraction of 20 mL volume using 2:1:1 acetone/EtOAC/cyclohexane with the addition of salts. After the centrifugation of the milk/solvent/salt mixture, approximately 15−16 mL of the upper solvent layer was recovered. The solvent formed the supernatant with milk solids concentrated as a plug at the interface separating the organic phase from the salt-saturated aqueous phase at the bottom of the centrifuge tube. This milk homogenate after centrifugation resembles a typical QuEChERS homogenate with fruit or vegetable matter. If the fat partitioned completely into the organic phase, then the fat content for 15−16 mL of extract should be equal to 75−80% of the fat content for 20 mL of whole milk. When four of the recovered organic solvent layers were concentrated and dried to constant weight, they gave a fat content for the recovered extract that was consistently between 0.48 and 0.53 g fat or 94−105% of the expected fat content (*34*), supporting this conclusion.

A second study was done with cream to investigate the partitioning of the fat in a single liquid extraction and contrasting it with the conventional liquid/liquid extraction with hexane/diethyl ether. The fat recovered from cream using the modified extraction of Sannino et al. (*18*) or LLE with the AOAC procedure is given in **Table 2**. The results in **Table 2** for cream clearly demonstrate that the single partition procedure provides an extraction of fat as efficient as either LLE or the optimized PSE procedure with far fewer steps.

Extraction and Cleanup of Milk and Cream. A schematic of the procedure is illustrated in **Figure 2**. GPC conditions were tested with pesticide standards dissolved in solvent. Complete recovery was obtained when collection began at 11 min (~55 mL). However, tests with fortified milk revealed that lower recoveries were found for BHC-δ, diazinon, nanochlor, and procymidone (25−50%) with the collection point set at 11 min. The pesticides appear to be eluting slightly earlier in the presence of coextracted lipids than when using standards in solvent only, so the collection point was adjusted by 1 min to 10 min. These pesticides are not listed in **Table 3** due to these lower recoveries found at two fortification levels tested with the later collection point (0.2 and 1 ppb).

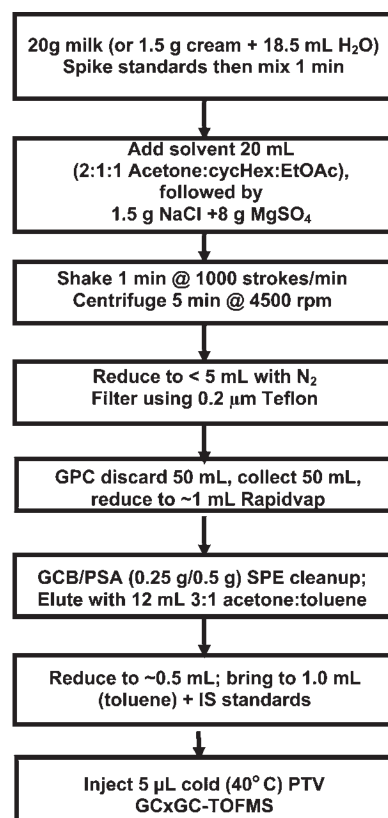

Figure 2. Flowchart for a multiresidue analysis of pesticides and POPs in milk and cream with a single extraction followed by GPC and SPE.

The collection point set at 10 min produced a pesticide fraction still containing approximately 2−3% of the fats extracted initially. Because the extract would need to be concentrated to at least 10 g/mL to reach the desired LOQs, further cleanup was required to obtain chromatography with stable retention times. An SPE column was employed containing PSA and carbon, because reports indicated that these materials should be effective at removing coextracted material from high-fat matrices (*5, 20, 33, 35*). After SPE cleanup using a 0.5 g PSA and 0.25 g of GCB, blank milk extracts were found to contain <1.5 mg of extracted residue from a 20 g milk aliquot. The final extracts were clean enough to produce stable retention times with both matrix-matched standards and the fortified milk.

Comprehensive Two-Dimensional GC×GC-TOFMS. Table 3 provides the target pesticide names, retentions on the two column types, quantitation mass, correlation coefficient obtained from a calibration with milk matrix-matched standards. The quantitation masses were usually chosen by the Chromatof software by selecting a unique mass which occasionally was changed by the operator. For example, the software chose m/z 81 for HE, but was changed manually to m/z 353. Similarities listed are those obtained with a 25 μg/L milk matrix standard used to build the

Table 3. Matrix-Matched Standard for 25 μg/L, Pesticide Common Name (POPs in Bold), CAS Registry Number, Quantitation Ion, GC Retention Times (First and Second Dimensions, 30 m VF5-msm and 2.2 m BPX-50) in Seconds, r^2, and Similarity to Library Spectrum[a]

compound name	CAS Registry No.	quant ion*m*/*z*	RT first col	RT second col	r^2	similarity	library
acenaphthene-d_{10}	15067-26-2	162	895	1.83	na	862	mainlib
aldrin	309-00-2	66	1483	2.19	0.999	870	replib
BHC-α	319-84-6	183	1129	2.05	0.999	901	mainlib
BHC-*β*	608-73-1	183	1189	2.21	0.999	899	mainlib
BHC-δ	319-86-8	181	1276	2.34	1.000	906	replib
chlordane-*cis*	5103-74-2	375	1684	2.35	0.998	843	pesticide1
chlordane-*trans*	5103-74-2	373	1648	2.33	0.998	793	pesticide1
chrysene-d_{12}	1719-03-5	240	2110	3.26	na	808	mainlib
dacthal	1861-32-1	301	1471	2.21	0.999	867	pesticide1
DDD-*o,p′*	53-19-0	235	1753	2.51	0.999	890	pesticide1
DDD-*p,p′*	53-19-0	235	1849	2.55	0.998	921	pesticide1
DDE-*o,p′*	3424-82-6	246	1645	2.38	0.999	907	replib
DDE-*p,p′*	3424-82-6	246	1732	2.37	0.999	855	replib
DDT-*o,p′*	789-02-6	235	1855	2.55	na	809	replib
DDT-*p,p′*	789-02-6	235	1951	2.58	0.999	869	replib
diazinon	333-41-5	137	1204	1.91	0.999	846	replib
dieldrin	60-57-1	79	1759	2.49	1.000	888	replib
endosulfan I	115-29-7	241	1687	2.42	0.999	903	pesticide1
endosulfan II	115-29-7	195	1849	2.71	0.999	881	pesticide1
endrin	72-20-8	263	1819	2.63	0.999	860	pesticide1
fonofos	944-22-9	109	1219	2.12	0.999	886	mainlib
heptachlor	76-44-8	100	1390	2.11	0.998	901	pesticide1
heptachlor epoxide	1024-57-3	353	1585	2.32	0.998	888	pesticide1
hexachlorobenzene	118-74-1	284	1141	2	0.998	886	pesticide1
lindane	319-85-7	183	1210	2.12	0.999	912	replib
mirex	2385-85-5	274	2257	2.91	0.999	813	pesticides1
nonachlor-*cis*	5103-73-1	407	1855	2.5	0.994	695	pesticides1
nonachlor-*trans*	39765-80-5	407	1690	2.26	0.999	853	mainlib
pentachloroaniline	527-20-8	265	1321	2.34	1.000	933	pesticide1
pentachlorobenzene	608-93-5	250	928	1.77	0.996	730	pesticide1
pentachlorobenzonitrile	20925-85-3	275	1207	2.16	0.999	896	pesticide1
pentachlorothioanisole	1825-19-0	294	1447	2.32	1.000	922	pesticide1
phenanthrene-d_{10}	1517-22-2	188	1246	2.31	na	917	pesticide1
procymidone	32809-16-8	96	1600	2.36	0.999	891	pesticide1
quintocene	82-68-8	237	1198	2.1	0.999	862	pesticide1
tecnazene	117-18-0	203	1000	1.91	0.999	887	pesticide1
tetrachloroaniline	3481-20-7	231	1045	2.01	0.999	906	replib

[a] Pesticide1 is a user-defined library using the Pegasus 4D spectra; mainlib and replib are from NIST05 library; na, not applicable.

calibration curve. Nearly all pesticides were found by the deconvolution software at the lowest standard concentration, 1 μg/L. Pentachlorobenzene at the lowest concentration sometimes had interference due to coeluting contaminants not originating from the milk. **Figure 3** provides an example of a two-dimensional separation for milk fortified at 10 μg/kg wet weight using a 30 m 5% phenyl (Sil- Silicon copolymer or Si-Arylene type) column in the first dimension and a 2.2 m 50% phenyl phase 0.1 mm i.d. column for the second-dimension column. A second set of columns using a 30 m 5% phenyl (non Si-Arylene type) column with a 50% phenyl second-dimension column with a wider diameter, 1.5 m × 0.15 mm i.d., gave an equally useful separation in 10 min less time with greater sample capacity. This column set was tested with repeated fortifications at 0.4 and 10 μg/kg milk (**Figure 4**). The later eluting pesticides were more effectively separated from coextracted fats and other nonpolar compounds on either column set. Remaining fats and other hydrocarbons eluted earlier than most pesticides in the second-dimension column, demonstrating the usefulness of the GC×GC technique.

Although the pesticide separations on both column sets were generally adequate, pentachlorobenzonitrile and fonophos coeluted on the HP5-ms with the shorter BPX-50 second column in fortified samples. We assumed that the change in the first-dimension retention time coupled with the larger oven offset produced the coelution, indicating that smaller oven offsets are desirable in this case. The wraparound observed for chrysene was reduced with larger offset, but was not close to being eliminated (**Figure 4**). The matrix-matched standards for this second column set found fonophos and pentachlorobenzonitrile at 896 and 1.42 s and at 893 and 1.42 s, respectively, separated by one modulation period. The second-dimension separation observed between pentachlorobenzonitrile and lindane with the smaller oven offset was eliminated (**Table 3**). The fortified sample's base peak for both pesticides, after modulation, was at 896 s. The second-dimension retention times were the same for both compounds, so they ended up with the same retention times in both dimensions. This did not happen with the first-column set with lindane and pentachlorobenzonitrile because these pesticides were separated by two modulations in the first dimension and the second-dimension rentention times were also different (**Table 3**; **Figure 3**). Because identification and confirmation are dependent on deconvolution of the pesticide from other pesticides and the matrix, sufficient separation and/or peak modulations are critical. Van der Lee et al. discuss the successful separation and deconvolution of 360 pesticides and PCB congeners in a single GC×GC analysis with a programmed offset from 10 to 40 °C, a faster 10 °C/min temperature ramp, but a longer 46 min analysis time (*22*) using a column set similar to the first one used in this study. Van der Lee used a proprietary nonpolar 30 m × 0.25 mm i.d. RTX-CL pesticide column

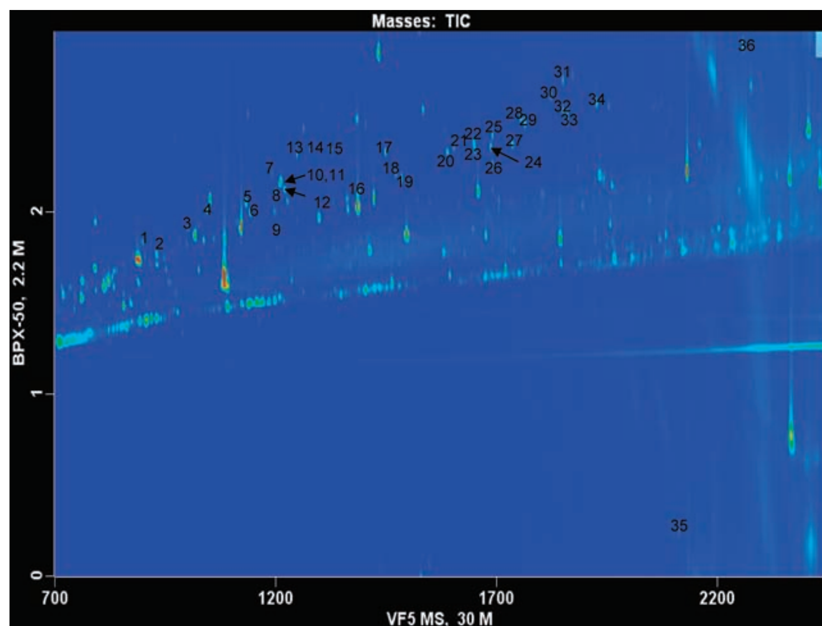

Figure 3. Comprehensive two-dimensional separation for pesticides and internal standards fortified in milk at 10 μg/kg using a 30 m × 0.25 mm i.d. 5% phenyl column (VF5-ms) and a 2.2 m × 0.10 mm i.d. 50% phenyl column (SGE BPX-50): (1) acenaphthene-d_{10}; (2) pentachlorobenzene; (3) tecnazene; (4) tetrachloroaniline; (5) BHC-α; (6) hexachlorobenzene; (7) BHC-β; (8) quintozene; (9) diazinon; (10) pentachlorobenzonitrile; (11) lindane; (12) fonofos; (13) phenanthrene-d_{10}; (14) BHC-δ; (15) pentachloroaniline; (16) heptachlor; (17) pentachlorothioanisole; (18) dacthal; (19) aldrin; (20) heptachlor epoxide; (21) procymidone; (22) DDE-*o,p′*; (23) chlordane-*trans*; (24) chlordane-*cis*; (25) endosulfan I; (26) nonachlor-*trans*; (27) DDE-*p,p′*; (28) DDD-*o,p′*; (29) dieldrin; (30) endrin; (31) endosulfan II; (32) DDD-*p,p′*; (33) nonachlor-*cis*; (34) DDT-*p,p′*; (35) chrysene-d_{12}; (36) mirex.

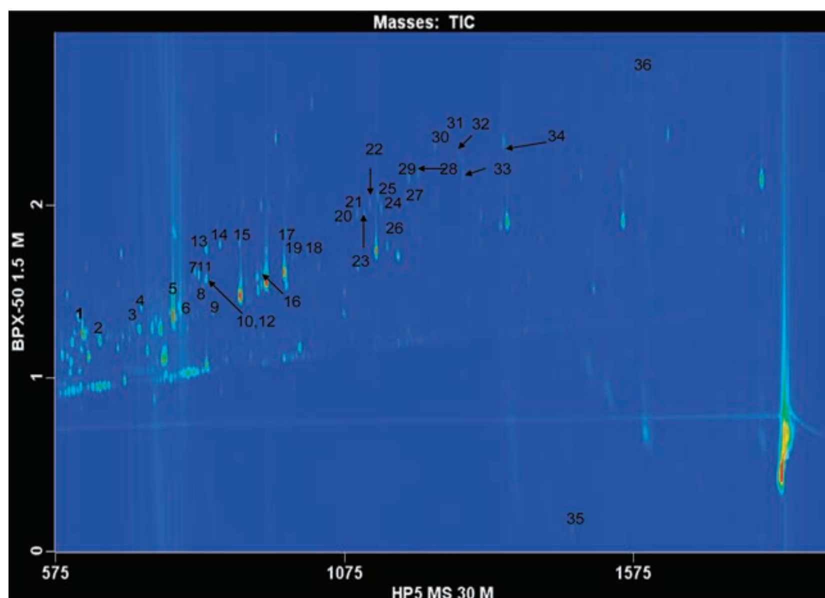

Figure 4. Comprehensive two-dimensional separation for pesticides and internal standards fortified in milk at 10 μg/kg repeated fortification using a 30 m × 0.25 mm i.d. 5% phenyl column (HP5-ms) and a 1.5 m × 0.15 mm i.d. 50% phenyl column (SGE BPX-50): (1) acenaphthene-d_{10}; (2) pentachlorobenzene; (3) tecnazene; (4) tetrachloroaniline; (5) BHC-α; (6) hexachlorobenzene; (7) BHC-β; (8) quintozene; (9) diazinon; (10) pentachlorobenzonitrile; (11) lindane; (12) fonofos; (13) phenanthrene-d_{10}; (14) BHC-δ; (15) pentachloroaniline; (16) heptachlor; (17) pentachlorothioanisole; (18) dacthal; (19) aldrin; (20) heptachlor epoxide; (21) procymidone; (22) DDE-*o,p′*; (23) chlordane-*trans*; (24) chlordane-*cis*; (25) endosulfan I; (26) nonachlor-*trans*; (27) DDE-*p,p′*; (28) DDD-*o,p′*; (29) dieldrin; (30) endrin; (31) endosulfan II; (32) DDD-*p,p′*; (33) nonachlor-*cis*; (34) DDT-*p,p′*; (35) chrysene-d_{12}; (36) mirex.

(Restek Corp.) rather than a nonpolar 5% phenyl phase as we did. Both van der Lee's column set and our column set were adjusted to provide nearly optimal flow rates for the second dimension while giving a slightly more optimal flow for the first dimension at 200 °C than would otherwise have occurred according to Beens et al. (*21*). It should be noted that neither pentachlorobenzonitrile nor fonophos is expected to be found in milk, and certainly not together. The pesticide POPs sometimes found in milk were separated easily (**Figures 3** and **4**).

Fortified whole milk recoveries at six levels analyzed in quadruplicate are reported in **Table 4**. Average recoveries for 29 pesticides, isomers, or metabolites were 77, 72, 73, 66, 77, and 84% for 0.2, 0.4, 1, 2, 10, and 50 μg/kg wet weights, respectively, and the average relative standard deviations were 10, 8, 7, 7, 3,

Table 4. Cow's Milk Recoveries for Pesticides (LOQ) at 0.2, 0.4, 1, 2, 10, and 50 μg/kg Wet Weight; [a]

pesticide name (LOQ)	0.2 (n = 4)	0.4 (n = 4)	1 (n = 4)	2 (n = 4)	10 (n = 4)	50 (n = 2)
aldrin (0.4)	69 ± 12	68 ± 5	69 ± 5	76 ± 7	72 ± 1	83 ± 1
BHC-α (0.2)	69 ± 6	57 ± 6	70 ± 6	72 ± 5	73 ± 4	87 ± 7
BHC-β + lindane (0.2)	68 ± 9	60 ± 5	62 ± 3	68 ± 9	71 ± 2	98 ± 5
chlordane-*cis* (0.4)	84 ± 12	69 ± 5	68 ± 4	63 ± 4	70 ± 1	84 ± 0.8
chlordane-*trans* (0.2)	82 ± 6	79 ± 4	74 ± 4	61 ± 6	61 ± 3	82 ± 3
dacthal (0.2)	81 ± 9	75 ± 6	75 ± 3	70 ± 3	76 ± 1	86 ± 1
DDD-*o,p′* (0.2)	52 ± 5	72 ± 4	67 ± 7	62 ± 6	72 ± 1	nd
DDD-*p,p′* + DDT-*o,p′* (0.2)	58 ± 10	67 ± 6	66 ± 8	62 ± 6	69 ± 3	93 ± 2
DDE-*o,p′* (0.2)	69 ± 8	73 ± 5	56 ± 7	63 ± 6	79 ± 2	88 ± 2
DDE-*p,p′* (0.2)	65 ± 13	80 ± 10	75 ± 7	64 ± 4	79 ± 1	102 ± 1
DDT-*p,p′* (0.4)	80 ± 18	79 ± 7	66 ± 8	64 ± 5	83 ± 2	77 ± 3
dieldrin (0.2)	90 ± 10	79 ± 11	87 ± 8	73 ± 6	85 ± 1	96 ± 4
endosulfan I (0.4)	110 ± 18	71 ± 11	73 ± 24	66 ± 9	80 ± 3	90 ± 0
endosulfan II (0.4)	43 ± 12	80 ± 10	73 ± 7	nd	70 ± 4	94 ± 0.5
endrin (0.2)	93 ± 6	78 ± 10	88 ± 13	74 ± 8	86 ± 0.6	94 ± 2
fonofos (0.4)	87 ± 13	86 ± 6	71 ± 2	67 ± 9	70 ± 2	80 ± 1
heptachlor (0.4)	64 ± 27	74 ± 9	71 ± 4	70 ± 6	76 ± 2	74 ± 1
heptachlor epoxide (0.4)	101 ± 9	84 ± 7	77 ± 3	76 ± 6	78 ± 2	79 ± 0
hexachlorobenzene (0.2)	76 ± 9	59 ± 12	74 ± 7	52 ± 10	79 ± 1	67 ± 1
mirex (0.2)	86 ± 5	70 ± 9	102 ± 11	72 ± 5	96 ± 6	119 ± 16
pentachloroaniline (0.2)	72 ± 6	75 ± 5	68 ± 4	59 ± 5	72 ± 4	78 ± 1
pentachlorobenzene (1)	bst	nd	58 ± 2	nd	73 ± 4	68 ± 3
pentachlorobenzonitrile (0.2)	76 ± 8	63 ± 8	63 ± 7	60 ± 9	68 ± 7	73 ± 3
pentachlorothioanisole (0.4)	87 ± 13	61 ± 4	62 ± 9	54 ± 3	66 ± 8	86 ± 2
quintozene (0.4)	83 ± 17	67 ± 8	92 ± 7	60 ± 9	100 ± 7	68 ± 2
tecnazene (0.2)	64 ± 2	nd	88 ± 4	74 ± 8	96 ± 6	64 ± 0.9
tetrachloroaniline-2,3,5,6 (0.2)	81 ± 5	76 ± 5	79 ± 4	64 ± 14	83 ± 5	68 ± 3
mean ± SD	77 ± 10	72 ± 8	73 ± 7	66 ± 7	77 ± 3	84 ± 3

[a] Mean ± RSD; nd, not deconvoluted; bst, below similarity threshold.

and 3%, respectively (**Table 4**). The pesticide recoveries were similar at each fortification level, although the relative standard deviation increased by 3.3 times as the concentration decreased from the highest spike to the lowest. The estimated LOQ is listed next to each pesticide name. **Table 4** reports fortifications at the action levels and at concentrations at least 5−10 times below the action levels for BHCs (300 μg/kg fat total BHCs), HE (50 μg/kg fat), and the sum of DDE, DDD, and DDT (1250 μg/kg fat) in milk. The pesticide recoveries were generally acceptable and averaged 75 ± 10% across all concentrations. For example, at 0.4 μg/kg recoveries for 32 analytes averaged 72 ± 7% with a range of 57−84%. In this example, tecnazene and pentachlorobenzene were not deconvoluted due to coeluting interference, so recoveries were not calculated.

DDD-*p,′p* and DDT-*o′,p* or BHC-β and lindane were sometimes deconvoluted as the same compound in fortified milk owing to their nearly identical spectra and very close elutions. Recoveries were usually > 70% except for hexachlorobenzene, pentachlorobenzene, pentachlorothioanisole, and pentachlorobenzonitrile, which have slightly lower recovery when GCB is used. At the LOQs, the average relative standard deviations were all < 15% (**Table 4**).

HE recoveries at 0.4, 1, 2, 10, and 50 μg/kg wet weight averaged 79 ± 4% (**Table 4**) corresponding to 12, 31, 62, 310 (n = 4) and 1550 (n = 2) μg/kg milk fat in the whole spike matrix. All fortified pesticides, metabolites, or isomers could be found automatically by deconvolution at the lower fortification concentrations except pentachlorobenzene. Some pesticides were not reported at certain concentrations, because they were either not found during deconvolution or were closely eluting with other isobaric pesticides (**Table 4**). The lowest two concentrations of the matrix standards for pentachlorobenzene were often not deconvoluted from the more abundant isomers of what the library search indicated were alkyl-substituted biphenyls or naphthalenes.

In March of 1982, the State of Hawaii Department of Health (DOH) recalled dairy products on Oahu due to contamination with HE used on pineapple leaves that had been fed to cows. On July 6, 1982, nearly 4 months after the initial recall, the DOH embargoed 120 plastic containers (45 lb each) of cream contaminated with HE that was still being sold from a food broker's warehouse. The DOH tests of 11 cream containers revealed they contained HE at levels of 620−710 μg/kg fat, over twice the action level at that time. In 2003, two samples were pulled from the cream and provided to a local laboratory and the U.S. FDA for future testing. In August 2003, the local (Hawaii) laboratory reported 250 μg/kg for HE and also found DDE-*p,p′* at 45 μg/kg wet weight. The HE concentrations were lower than those reported in Hawaii in 1982 (*36*). The HE concentration exceeded the allowable level for disposal at the Oahu landfill. The cream was declared a hazardous waste and properly shipped and disposed of in a facility in Washington State in November of 2003. The Hawaii Heptachlor Research and Education Foundation paid for the disposal. The other sample was held at the U.S. FDA CFSAN until 2009, when it was tested.

The Hawaiian cream sample when thawed revealed that the solid and aqueous phases of the sample had largely separated. HE action levels are evaluated on a lipid-adjusted basis so only the lipid content of a test portion is relevant in this case. The cream was otherwise in good condition for such an old sample having been stored at −40 °C for 27 years. The cream solids were sampled for analysis in triplicate. The test portions were combined with 18.5 mL of HPLC-grade water and analyzed through the procedure like a whole milk test portion. Approximately 19 mL of the 20 mL of extraction solvent added was recovered. An 8 mL aliquot was evaporated to < 5 mL for cleanup on GPC. A 4 mL aliquot of the extract was evaporated, dried, and weighed for lipid content. The three test portions were 72 ± 1% fat.

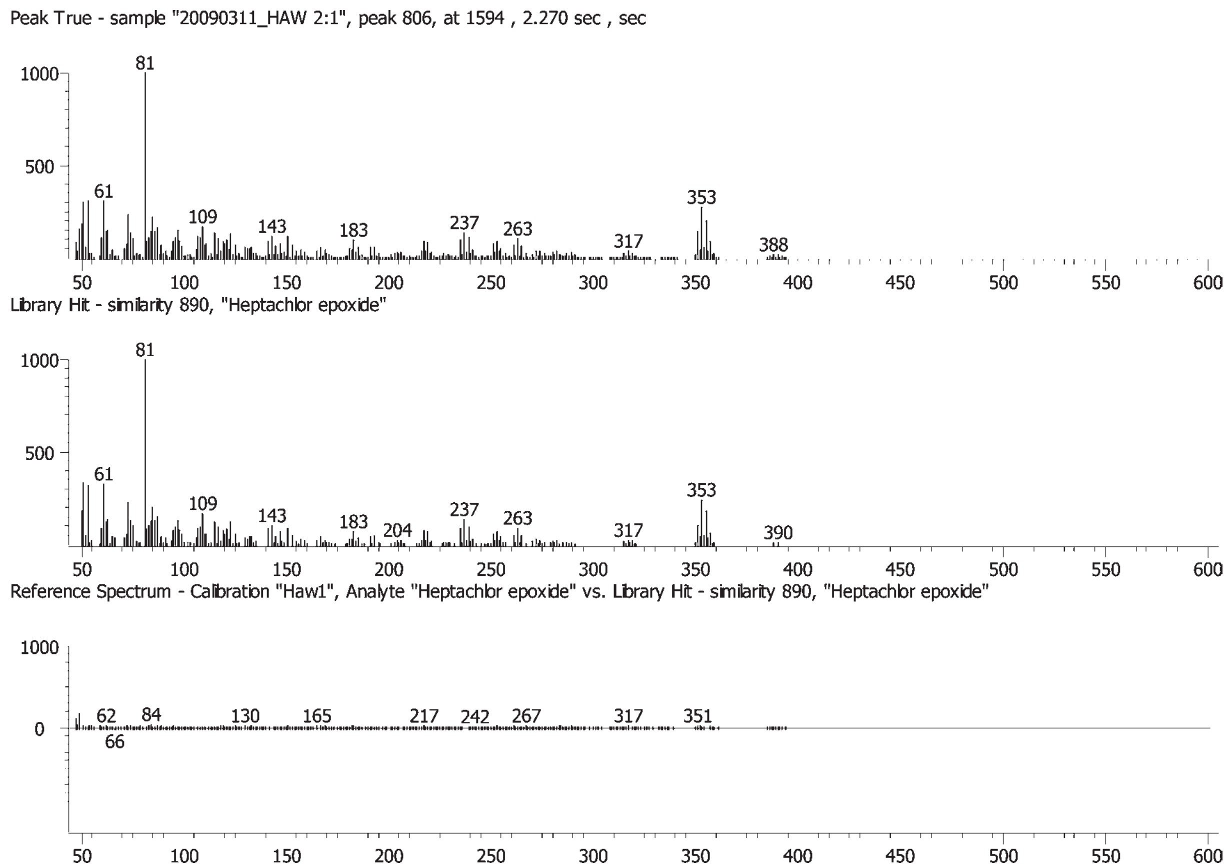

Figure 5. Heptachlor epoxide (HE) spectrum found in 1982 Hawaiian heavy cream test sample. Top spectrum is the deconvoluted spectrum of HE from the heavy cream with retention times of 1594 s D^1 and 2.27 s D^2. Middle spectrum is HE from a user-defined library of the LECO Pegasus 4D TOFMS, and the bottom spectrum is the difference spectrum. Matrix-matched HE standard retention times were 1591 s D^1 and 2.27 s D^2.

Table 5. Hawaiian Cream Collected on Oahu in 1982 during a Cream Embargo[a]

	test portion			
compound name	1	2	3	mean ± SD
heptachlor epoxide	410	380	350	380 ± 29
DDE-*p*,*p*′	63	85	55	68 ± 16

[a] Concentrations are in μg/kg fat. HE action level = 50 μg/kg fat.

Deconvolution of each triplicate GC×GC-TOFMS data file revealed only HE and DDE-*p*,*p*′. No other pesticide or PCB was found above the LOQs, although these other pesticides and POPs are known to be recovered in the cleanup (*37*). The deconvoluted "peak true" spectrum of HE is presented in **Figure 5** along with the hit to the pesticide1 user-defined library (890 similarity), and difference spectrum matched the retention times on the primary and secondary columns of the matrix-matched standard (not shown), which were 1591 and 227 s. HE and DDE-*p*,*p*′ were found at an average concentration of 380 and 68 μg/kg fat, respectively, based on the matrix-matched milk standard curve (**Table 5**). HE was also calculated using the method of additions, and extrapolated concentration was found to be 330 μg/kg fat.

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