

Assessment of chlorinated pesticides and polychlorinated biphenyls in adipose breast tissue using a supercritical fluid extraction method

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A precise and highly reproducible analytical method was developed for the assessment of organochlorinated pesticide and polychlorinated biphenyl residues in adipose tissue (≥ 50 mg). The method can be utilized for epidemiological studies on the significance of these environmental pollutants in the etiology of breast cancer. Supercritical fluid extraction (SFE) with CO₂ and modified CO₂ (addition of 5% dichloromethane) is employed to remove incurred pesticide residues from adipose tissues that have been surgically removed from breast cancer patients and controls. An alumina sorbent, placed in the extracting vessel together with a specimen, removes the bulk of co-extracted lipids; a subsequent purification of the SFE extracts by column chromatography on alumina removes the remaining traces of lipids that would interfere with the gas chromatographic analysis with electron capture detection. The method was tested by analyzing a Certified Reference Material 430 pork fat with known amounts of pesticide residues that are commonly found in fat or in foods with a high fat content. The recoveries of analytes ranged from 73.4% for endrin to 115% for α -, β - and γ -hexachlorocyclohexane, hexachlorobenzene and dieldrin, with standard deviations of 4–12% for individual analytes. The analysis of adipose tissue for organochlorinated compounds on the basis of this new method suggested that the pesticide levels were higher in breast cancer patients than in controls. However, the small number of samples analyzed in this study ($n = 5$, both groups) precludes definitive conclusions. The most abundant compounds in both cases and controls were *p,p'*-DDE (379 ± 286 and 160 ± 149 p.p.b.) and PCB (223 ± 145 and 124 ± 65.7 p.p.b.), followed by the termiticide chlordane residues oxychlordane and *trans*-nonachlor.

Introduction

Breast cancer is the second leading cause of death from cancer among American women. Overall, breast cancer incidence has increased significantly in recent years and clusters of unusually high rates have been observed in some areas (1,2). Established

risk factors for breast cancer, primarily late age at first parity, early menarche, late menopause and family history, appear to be involved in ~30% of all cases (3). Estrogens, cholesterol epoxides and dietary fats are all thought to be significant factors in the development of breast cancer (4,5). Experimental evidence revealed that some environmental agents, such as specific chlorinated organics, affect estrogen production and metabolism, and thus function as xenoestrogens (6). The first suggestions of a relationship between elevated chlorinated pesticide concentrations in adipose tissues and induction of carcinoma at various sites were presented in 1968 (7). To test this hypothesis, Wassermann *et al.* studied the distribution of organochlorinated compounds (OCC*) and polychlorinated biphenyls (PCB) in neoplastic and in adjacent, apparently normal breast tissue obtained from women hospitalized in São Paulo, Brazil (8). Even though the data were based on only a few cases ($n = 9$; controls $n = 5$), the authors concluded that the levels of OCC and PCB in the lipids of malignant breast tissue were significantly elevated over levels in adjacent breast tissue. As for the distribution of OCC in adipose tissue, the levels of some compounds were higher in breast cancer patients while the levels of others were elevated in controls. Falck *et al.* also reported that the levels of some OCC—primarily dichlorodiphenylethylene (DDE), dichlorodiphenyltrichloroethane (DDT) and PCB—in adipose tissues of 20 breast cancer patients in Hartford, CT, were significantly higher than in 20 controls (9). Mussalo-Rauhamaa *et al.* (10), studying the distribution of OCC and PCB in adipose tissue of Finnish women, found that only the concentrations of β -hexachlorocyclohexane (β -HCH) were significantly higher in breast cancer patients ($n = 44$) than in controls ($n = 33$). Dewailly and co-workers (11) went one step further, examining the levels of OCC in adipose tissue of women with benign breast disease ($n = 17$) and with breast cancer (both with and without estrogen receptors) ($n = 9$ each group). In estrogen receptor-positive (ER-positive) cases DDE and PCB congener 99 levels were substantially higher than those in controls. Mean adipose tissue concentrations of OCC in ER-negative cases were generally lower than those in controls. These results suggest that women with hormone-responsive breast cancer (ER-positive) have higher DDE body burden. This, in turn, supports the hypothesis that exposure to estrogenic OCC may affect the incidence of hormone-responsive breast cancer.

To date, two large case-control studies, nested within cohorts which were conducted in New York City and the San Francisco Bay area, have examined the relationship between exposure to organochlorinated compounds (DDE and PCB) and the risk of breast cancer. The results were inconsistent (12,13); while Wolff *et al.* found a positive relationship between breast cancer risk and serum levels of DDE (the major metabolite of DDT) in 58 cases and 171 controls, Krieger *et al.*, who analyzed serum from 150 cases and 150 matched controls for organochlorine content, concluded that the data do not support the hypothesis that exposure to DDE and PCB increases the risk of breast cancer. Clearly, further studies on still larger samples are

*Abbreviations: OCC, organochlorinated compounds; PCB, polychlorinated biphenyls; *p,p'*-DDE, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene; *p,p'*-DDT, 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane; ER-positive, estrogen receptor-positive; ER-negative, estrogen receptor-negative; SFE, supercritical fluid extraction; GC-ECD, gas chromatography with electron capture detection; HCB, hexachlorobenzene; HCH, hexachlorocyclohexane; HCE, heptachlor epoxide; TNC, *trans*-nonachlor; OXC, oxychlordane; HC, heptachlor; CRM 430, Certified Reference Material 430; DCM, dichloromethane.

required to resolve whether these compounds contribute to breast cancer risk.

While it is obviously possible to measure levels of lipophilic OCC in serum, we chose to assess these compounds in adipose tissue for several reasons. First, the levels of OCC are 10–100 times higher in adipose tissue, allowing more reliable quantification and determination of those analytes that are present in lower amounts than the predominant DDE and PCB. Secondly, OCC in serum are much more sensitive to changes in body weight and overall body status than OCC in adipose tissue. For instance, it has been reported that serum levels of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin are lowered over time due to changes in body composition with age, independent of the expected decomposition of this compound (14). In other words, changes in the health status of an individual can lead to changes in the migration of OCC from fat stores into serum.

To provide an analytical tool for the analysis of a large number of adipose tissue samples as required in epidemiologic studies, we deemed it important to develop a method that would increase the sample output and enable us to determine accurately and reproducibly the p.p.b. concentrations of analytes in specimens as small as 50 mg. The method developed by us is based on the supercritical fluid extraction (SFE) of incurred pesticide residues from adipose tissue in the presence of an alumina sorbent, to remove the bulk of co-extracted lipids. Column chromatography on alumina follows to remove the traces of lipids that would interfere with a subsequent OCC analysis by gas chromatography with electron capture detection (GC-ECD). It has been documented that the pesticide recoveries by SFE from various matrices, including fat, were similar, or often higher than those obtained by traditional solvent extraction (15–18). Therefore, the method described here will enable us to assess qualitatively and quantitatively not only the most abundant OCC, such as DDE and PCB, but also those analytes that are present in minute concentrations.

Materials and methods

Patient population

The incidence rate for breast cancer in Nassau County on Long Island is significantly higher than incidence rates for New York State and for the United States as a whole (19,20). We therefore established collaborations with major medical centers in this area. Preparatory to a case-control study of breast cancer in Long Island, a pilot study was undertaken to establish working arrangements at collaborating hospitals for identifying breast cancer patients, collecting and storing specimens, transporting them to our laboratories, and performing the requisite analyses. To date, 47 samples and pathological reports have been received from Long Island Jewish Medical Center, New Hyde Park, NY. Each sample of surgically removed adipose tissue, which was adjacent to the tumor, was placed in a labelled, prewashed (detergent, water, acetone and *n*-hexane), 20 ml glass scintillation vial and stored at -20°C in a hospital freezer, until a sufficient number of them were collected for analyses. To test the applicability of a new analytical method, five samples from breast cancer patients and five samples from hospital controls (average age 58.8 and 49.8 years respectively) were analyzed.

Materials, reagents, and standards

Residual levels of the following OCC and PCB were determined in adipose tissue: hexachlorobenzene (HCB; fungicide), hexachlorocyclohexane isomers (α -, β - and γ -HCH; insecticides), the residues of cyclodiene compounds, such as the termiticide chlordane [heptachlor epoxide (HCE), *trans*-nonachlor (TNC), and oxychlordane (OXC)], the insecticide aldrin (dieldrin and endrin), then DDT and its metabolite DDE (insecticides often used to fight nematodes or mosquitos), and PCB, expressed as total (industrial chemicals, electric insulating fluid). A chlorinated pesticide mixture (the concentrations of individual OCC in iso-octane ranged from 25 to 260 p.p.m.) as well as individual OCC (100 p.p.m. in methanol), including heptachlor (HC), which was used as an internal standard, were purchased from Alltech Associates, Inc., Deerfield, IL. Prior to analyses by GC-ECD, the pesticide mixture was

diluted 500 times (the working concentrations were 0.05–0.52 ng/ μl). PCB mixtures (Aroclor 1248, 1254 and 1260; 35 p.p.m. in iso-octane) were purchased from Chemical Research Supplies, Addison, IL and chlordane, OXC and TNC (100 p.p.m. in methanol) from AccuStandard, Inc., New Haven, CT. All chemicals and solvents were analytical reagents of the highest purity from J.T.Baker Chemical Co., Phillipsburg, NJ and Fischer Scientific Co., Fair Lawn, NJ. Alconox powder detergent used for washing glassware was purchased from Alconox, New York, NY. 4-Methoxyazobenzene, which was used to determine the solvent volume required to elute OCC, but not lipids, from SFE extracts during the clean-up on the alumina column, was purchased from Eastern Chemical, Hauppauge, NY. Neutral alumina (20–200 μm particle size), which was used during SFE for the *in situ* removal of fat and for a column chromatography clean-up, was purchased from ICN Biomedicals, Irvine, CA. Certified Reference Material 430 (CRM 430, pork fat containing known amounts of OCC), which was needed for the SFE method development, was purchased from the European Community Bureau of Reference, Brussels, Belgium.

Apparatus

Extraction of OCC from CRM 430 pork fat and human adipose tissue was performed on the PrepMaster Integrated SFE system (Suprex Corporation Pittsburgh, PA) with AccuTrap SFE Collecting Module, equipped with a Dura-Flow restrictor (2.0 ml/min) and Modifier Pump. Carbon dioxide of SFC grade was purchased from Cryodyne Technology Inc., Chester, CT. High-pressure gas purifiers were obtained from Alltech Associates, Inc. To eliminate GC-ECD background, it is essential to use exclusively Teflon tubings, valves and septa in all steps of the analytical procedure. GC-ECD analyses were performed with a Hewlett-Packard GC Model 5890, equipped with a Hewlett-Packard 19233 ^{63}Ni ECD (Hewlett-Packard Co., Wilmington, DE). The analyses were performed by splitless injection (purge delay time 1.0 min) on a DB-5 fused silica capillary column (a 30 m \times 0.32 mm i.d., 1.0 μm film thickness; J&W Scientific, Folsom, CA).

Methods

The assay of OCC in adipose tissue consisted of (i) the extraction by supercritical CO_2 , followed by extraction with CO_2 modified with 5% dichloromethane (DCM), and by an *in situ* removal of the bulk of fat on a partially deactivated neutral alumina sorbent; (ii) the clean-up of SFE extracts by adsorption column chromatography to remove the remaining traces of fat; and (iii) GC-ECD analyses.

SFE. At a developmental stage of the method, OCC were extracted from the CRM 430 pork fat with the known amounts of pesticide residues [HCB, 392 p.p.b.; α -HCH, 140 p.p.b.; β -HCH, 259 p.p.b.; γ -HCH (lindane), 500 p.p.b.; β -HCH, 109 p.p.b.; dieldrin, 124 p.p.b.; endrin, 20 p.p.b.; 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane (*p,p'*-DDT), 3400 p.p.b.; and 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (*p,p'*-DDE), 820 p.p.b.] that are most frequently found in fat or in foods with a high fat content. The certified values for each pesticide, based on conventional extraction methods, represent the means of nine sets of results obtained in different laboratories (21). The pesticide residues were extracted from fat in the presence of an alumina sorbent to remove co-extracted lipid material (fatty acids, diglycerides and triglycerides as well as minor lipid components such as sterols) that can interfere in the subsequent gas chromatographic analyses (15). The fat was placed in a stainless-steel extraction vessel in a sandwich-like fashion as described by King *et al.* (16): a filter paper, prewashed with methanol and *n*-hexane to remove possible contaminants, was rolled up to fit the vessel, 1 g of neutral alumina, Brockmann activity II, (deactivated with 3% water, w/w), was placed on the bottom, ~200 mg of pork fat were added, and 1 g of alumina was placed on top. The pork sample was spiked with 20 μl of internal standard solution (0.5 p.p.m. heptachlor in iso-octane) to monitor the efficiency of the analysis. The three-stage SFE was carried out in both the static and the dynamic mode (10 and 20 min respectively, each stage) at 50°C and 330 atm; CO_2 was used for the first two stages of extraction and the modified CO_2 (plus 5% DCM) was employed in the final extraction stage. The modification of CO_2 was necessary to improve the overall recovery (Table I). The extract was flushed from the AccuTrap (40°C) with cyclohexane into 4 ml vials. In the first two stages 8 ml cyclohexane extract were obtained (F1) and 4 ml in the third stage (F2). The extracts obtained with CO_2 and with the modified CO_2 were collected and processed separately to determine the effect of DCM on the overall OCC recoveries.

The extracts of organochlorinated compounds, including PCB from adipose tissues (sample range 64–890 mg) of breast cancer cases and controls, were prepared by the procedure that gave the highest recoveries of analytes from CRM 430 pork fat. Before the adipose tissue extractions by SFE, a blank experiment was carried out under the same conditions to ensure that there is no background response stemming from solvents, reagents, or other materials that may interfere with the GC-ECD analysis.

Table I. Recoveries of individual OCC from CRM 430 pork fat

	Recovery (%)								
	α -HCH	HCB	β -HCH	γ -HCH	HCE	<i>p,p'</i> -DDE	Dieldrin	Endrin	<i>p,p'</i> -DDT
SFE (F1): CO ₂									
Average (5) ^a	92.0	99.9	89.0	89.2	85.5	79.3	102	60.2	74.8
SD	12.0	10.5	6.9	12.2	10.0	4.0	8.3	9.0	8.4
SFE (F2): CO ₂ + 5% DCM									
Average (3) ^a	7.5	14.8	20.5	16.3	19.4	13.0	ND	13.2	16.8
Total recovered (F1 + F2)	99.5	115	110	106	105	92.3	102	73.4	91.6

ND, not detected.

^aNumbers in parentheses indicate the number of repeat analyses.

Purification. Purification of OCC is most often based on column chromatography utilizing florisil (60–100 mesh) as an adsorbent, and *n*-hexane as an eluent (10,22), or on gel permeation chromatography (9,23). In our hands, the clean-up of SFE-extracts on neutral alumina Brockmann activity II–III (deactivated with 5% H₂O, w/w) was more satisfactory than on florisil in respect to the recovery rates of individual compounds, especially epoxides (HCE, dieldrin and endrin) and β -HCH, which were higher, while the levels of the impurities that interfere with GC–ECD analysis were lower. The adsorption chromatography was performed on a glass column (10.5×250 mm, with a top reservoir) controlled by a Teflon stopcock. The column was filled with 10 g alumina and topped with a 2–3 mm layer of anhydrous sodium sulfate. Following column conditioning with cyclohexane, sample application, and OCC elution first with 45 ml cyclohexane, then with 45 ml of cyclohexane containing 10% DCM (v/v), the eluate (90 ml) was concentrated to ~1 ml under vacuum and transferred quantitatively into a 1.5-ml GC vial. It was dried with nitrogen, and redissolved in an ECD grade iso-octane. The required volume of the eluent (90 ml) was determined in a separate experiment by dissolving 0.1–0.2 g 'blank' fat in 1 ml dye solution (0.3 g *p*-methoxyazobenzene in 100 ml cyclohexane) and applying this solution onto the alumina column (24). Visible dye separates from the fat as it travels downward in a bright orange band. When this band was eluted, the solvent volume was measured. The determined solvent volume is usually sufficient to elute fat-free organochlorinated compounds.

Analysis. Gas chromatography with mass spectrometry (GC–MS) or GC–ECD are the methods of choice in OCC analysis. Mussalo-Rauhamaa *et al.* analyzed adipose tissue for OCC by GC–MSD (mass selective detector) in the selected ion monitoring (SIM) mode (10). The limit of detection for a single OCC was 10 μ g/kg fatty tissue (10 p.p.b.) and 50 p.p.b. for PCB. The determination of OCC by GC–EC has been described extensively in the literature (9,22,25). Analyses of OCC in this preliminary study were performed by GC–ECD. The compounds were separated on a DB-5 fused silica capillary column. The oven temperature was programmed from 80°C (1 min hold) to 190°C at 30°C/min, then to 270°C (15 min hold) at 2°C/min, and finally to 290°C (5 min hold) at 15°C/min; the injector temperature was 250°C, the detector temperature was 320°C. Helium was the carrier gas at a flow rate of 1–2 ml/min; nitrogen was used as a make-up gas with a flow rate of 60 ml/min. Quantitative data were ascertained by external calibration with the pesticide mixture (0.05–0.52 ng/injection) and Arochlor 1260 mixture (0.7 ng/injection). Arochlor 1260 resembles most closely the general PCB pattern in human adipose tissues. Aldrin was often used as an internal standard for quantification (9,16). However, in our experiments both CRM 430 pork fat and human adipose tissues contained a compound which eluted at the very same time as aldrin, thus disqualifying aldrin as a suitable analytical standard. Instead of monitoring the efficiency of the analysis with aldrin, adipose tissues were spiked with 20 μ l of internal standard solution (0.5 p.p.m. heptachlor in iso-octane) prior to extraction.

Results and discussion

Table I shows the recoveries of individual OCC from CRM 430 pork fat in an analysis based on SFE with column chromatography clean-up and a GC–ECD system. The total recoveries of all compounds by supercritical CO₂ and modified CO₂ (plus 5% DCM) ranged high, being 73.4% for endrin and 92–115% for other OCC. The modification of CO₂ with DCM enhanced the average recovery by 15.7%. The recoveries are

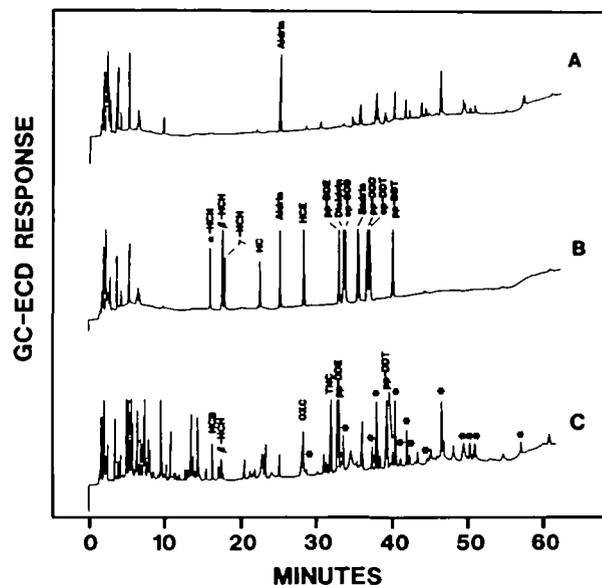


Fig. 1. GC–ECD chromatograms of (A) Arochlor 1260 standard; (B) the organochlorine pesticide mixture; and (C) the SFE extract of adipose tissue from a breast cancer patient. The asterisks in (C) indicate Arochlor 1260 peaks.

>100% for some analytes because the certified values for each OCC in CRM 430 pork fat represent the means obtained with the conventional solvent extraction method in nine different laboratories with the standard deviation for each compound varying significantly between laboratories.

The most critical step in the assessment of OCC in adipose tissue is a complete removal of the lipids from tissue extracts. Even though the SFE with the *in situ* purification on alumina removes the majority of fat (up to 99.8%; 16), the additional purification of the SFE extracts by column chromatography appeared to be mandatory to remove the remaining traces of lipids, because these can be detrimental to GC–ECD analysis. In this study, the most satisfactory results were obtained by adopting a micro-alumina column method (24) for the purification of the SFE extracts. All organochlorinated constituents of CRM 430 pork fat, with the exception of β -HCH, HCE, dieldrin and endrin, were recovered quantitatively, and free of fat, from the alumina column (10 g; Brockmann activity II–III) with cyclohexane as an eluent. To elute β -HCH, a lindane metabolite frequently found in human adipose breast tissues (10,11), and polar epoxides, cyclohexane had to be

Table II. Levels of OCC and PCB in adipose tissue of breast cancer cases and controls

ID	Age (years)	Estrogen and progesterone receptor status	ng/g net weight							Total
			HCB	β -HCH	OXC	TNC	<i>p,p'</i> -DDE	<i>p,p'</i> -DDT	PCB	
<i>Breast cancer case patients</i>										
1	72	ER-/PR-	24.2	9.1	32.5	26.6	58.5	ND	217	368
2	76	ER-/PR-	11.5	14.5	32.7	45.0	386	13.5	390	896
3	41	ER+/PR-	9.1	ND	6.2	7.0	156	ND	67.2	245
4	40	ER+/PR+	11.0	13.0	16.5	19.5	410	11.7	59.9	542
5	65	ER+/ER+	24.5	26.1	75.6	50.6	884	28.2	384	1473
		Average	16.1	12.5	32.7	29.7	379	10.7	224	705
		SD	6.8	8.5	23.7	16.1	286	10.4	145	442
<i>Control subjects</i>										
6	37		7.6	9.0	ND	4.7	87.3	4.4	67.5	180
7	66		5.1	ND	11.2	11.2	62.3	ND	150	240
8	59		8.7	18.2	19.6	20.9	92.7	3.8	131	297
9	43		23.6	23.4	47.4	66.2	457	12.2	228	858
10	44		5.1	ND	5.8	8.8	102	ND	42.0	164
		Average	10.0	10.1	16.8	22.4	160	4.1	124	348
		SD	6.9	9.5	16.6	22.5	149	4.5	65.7	259

ND, not detected.

modified with 10% DCM. In summary, 45 ml cyclohexane followed by 45 ml cyclohexane containing 10% DCM have to be used to recover quantitatively all OCC and PCB from SFE extracts by chromatography on the alumina column.

A representative GC-ECD chromatogram of the SFE extract of adipose breast tissue is shown in Figure 1C. The asterisks identify those peaks that have the same retention time as Aroclor 1260. Adipose tissue from both breast cancer patients and controls have the same profile of chlorinated pesticides and PCB: all samples contained measurable levels of HCB, β -HCH, OXC, TNC, *p,p'*-DDE, *p,p'*-DDT and PCB expressed as Aroclor 1260. Although the quantitative analysis revealed the levels of OCC and PCB to be higher in cases than in controls (Table II), the small number of samples analyzed in this study ($n = 5$, both groups) does not allow us to draw definitive conclusions about the role of OCC in breast carcinogenesis. The most abundant OCC both in cases and controls was *p,p'*-DDE, followed by PCB, and two chlordane residues, OXC and TNC. No detectable levels of HCE, dieldrin and endrin were found in any of the fat samples. The mean concentrations of the analytes in this study appear somewhat lower than reported elsewhere (9-11). In this exploratory study with the limited sample size it was also not possible to test the hypothesis of Dewailly *et al.* (11) that women with ER-positive breast cancer carry a higher organochlorine body burden than women with ER-negative breast cancer or than controls, nor could we answer the question whether the age differences between cases and controls have something to do with the higher OCC levels in cases, or whether older people *per se* retain more pesticides in adipose tissue. However, we plan to undertake a large-scale study in which ER and other factors will be considered.

In summary, a precise and highly reproducible method has been developed based on SFE, column chromatography on neutral alumina and GC-ECD for the assessment of OCC and PCB in adipose tissue. This method aids epidemiological evaluations related to studies of environmental factors and breast cancer. The method is quantitative, allowing the analyses of organochlorinated compounds in specimens as small as 50 mg.

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