

## Analytic Considerations for Measuring Environmental Chemicals in Breast Milk

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The presence of environmental chemicals in human breast milk is of general concern because of the potential health consequence of these chemicals to the breast-fed infant and the mother. In addition to the mother's exposure, several features determine the presence of environmental chemicals in breast milk and their ability to be determined analytically. These include maternal factors and properties of the environmental chemical—both physical and chemical—such as its lipid solubility, degree of ionization, and molecular weight. Environmental chemicals with high lipid solubility are likely to be found in breast milk; they include polyhalogenated compounds such as polychlorinated biphenyls, polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans, organochlorine insecticides, and polybrominated diphenylethers. These fat-soluble chemicals are incorporated into the milk as it is synthesized, and they must be measured in accordance with the fat content of the milk to allow for meaningful comparisons within an individual and among populations. Although the analytic approach selected to measure the environmental chemical is predominantly determined by the characteristics of the chemical, the concentration of the chemical in the milk sample and the existence of structurally similar chemicals (e.g., congeners) must be considered as well. In general, the analytic approach for measuring environmental chemicals in breast milk is similar to the approach for measuring the same chemicals in other matrices, except special considerations must be given for the relatively high fat content of milk. The continued efforts of environmental scientists to measure environmental chemicals in breast milk is important for defining the true contribution of these chemicals to public health, especially to the health of the newborn. Work is needed for identifying and quantifying additional environmental chemicals in breast milk from the general population and for developing analytic methods that have increased sensitivity and the ability to speciate various chemicals. *Key words:* analytic, chemical, environment, human breast milk, measurement, toxicant. *Environ Health Perspect* 110:A317–A324 (2002). [Online 13 May 2002]

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Breast milk is unique as a matrix for biomonitoring because, in addition to serving as a matrix for the many uses of biomonitoring, it also serves as a food source for a segment of the human population; thus, the analyses of breast milk for environmental chemicals as well as for nutrients are of wide scientific interest. One of the earliest reports of the measurement of an environmental chemical in breast milk was by Laug et al. in 1951 (1). They reported that the breast milk from 32 women from the general population of Washington, DC, contained 1,1,1-trichloro-2, 2-bis(4-chlorophenyl)ethane (*p,p'*-DDT or DDT) at an average concentration of 0.13 ppm. Laug et al. (1) attributed the primary source of DDT to their diet. Over the years, many more chemicals have been measured in human breast milk, our understanding of the interaction between lactation and exposure to environmental chemicals has grown, and our analytic methods have become more sophisticated. Because the fat content of milk is relatively high, most of the chemicals that have been monitored in milk are those that have high lipid solubility, in particular, polyhalogenated chemicals. These chemicals tend to

degrade slowly in the environment, to bioaccumulate and bioconcentrate in the food chain, and to have long half-lives in humans. Certain adverse health and reproductive outcomes have been attributed to these chemicals in laboratory animals and in wildlife, as well as in humans. Therefore, public health officials, environmental regulators, and scientists are concerned about their sources, their presence in our ecosystems and in people, and finally the relation between exposure and adverse health outcomes. Scientists develop and apply methods to measure these chemicals in human specimens, such as breast milk, and also in other matrices, both environmental and biological. These methods present challenges, such as the need for overcoming the relatively high fat content of milk while still maintaining all of the characteristics of state-of-the-art analytic methods. In this paper we note those characteristics and means of ensuring that they are met; we also describe in summary how breast milk is made, how environmental chemicals are incorporated into the milk, and factors that influence the levels of these chemicals in milk.

### Incorporation of Environmental Chemicals into Breast Milk

Following human exposure, environmental chemicals can be absorbed into the bloodstream by three routes: ingestion, inhalation, and dermal contact. These chemicals circulate in the bloodstream, either bound to carrier proteins such as albumin and lipoproteins or in their free form, and distribute among tissue compartments throughout the body (2). Initially, the rate of distribution of chemicals within the body is a function of tissue perfusion, which is the rate of blood flow through the various tissues. Highly vascular organs accumulate the chemicals first. Then, as equilibrium states are reached, the chemicals redistribute, and chemicals with high lipid solubility concentrate in tissues with higher fat content, such as adipose tissue, brain, liver, kidney, and, in the case of lactating women, breast milk.

In lactating women, lactogenesis begins about 40 hr after the birth of their offspring. During the first 3–5 days after delivery, the milk is low in volume and in fat (lipid) content (2.9%) and is called “colostrum.” Over the next 2–6 weeks, the transitional milk matures and increases in fat content to about 4%. The lipids are important for infant brain development; the major class of lipids in milk is the triglycerides, which are made from fatty acids such as arachidonic and docosahexaenoic acids (3). Breast milk is made up of several other components including carbohydrates, proteins, and minerals, especially calcium. Milk is synthesized in the mammary alveolar gland; to synthesize milk, milk components and their precursors pass through a membrane that separates the blood flowing in capillaries from the alveolar epithelial cell of the breast. However, during this process certain environmental chemicals present in the blood also can pass through the membrane and be incorporated into the breast milk at concentrations comparable to the

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chemicals' levels in other fatty compartments in the body (4,5). The most common mechanism for the passage of environmental chemicals is passive transport, which in general allows passage of lipophilic components of molecular weight < 800 Da; thus, lipid solubility of a chemical is a primary factor for its incorporation into breast milk. Factors that affect the lipophilic character of a chemical include its chemical structure and its degree of ionization (pKa) in the body compartments. For example, in general, halogens increase the lipophilic nature of a chemical. Also, chemicals in their nonionized state are more lipophilic than when in their ionized state and hence are more likely to diffuse into breast milk when in their nonionized state. Because the pH of plasma is 7.40, weakly acidic chemicals tend to exist primarily in their ionized form and thus are less likely than weak bases to pass through the membrane and into milk, which has a pH of 7.0–7.25 (6).

However, the passive transport mechanism for lipid-soluble chemicals is not the only mechanism for chemicals to cross cell membranes. For example, low molecular weight (< 200 Da) water-soluble chemicals can cross cell membranes with the bulk transfer of water. On the other hand, chemicals of high molecular weight (> 800 Da) tend not to pass through the membrane and likely do not enter breast milk to a measurable degree. Also, chemicals (e.g., heavy metals) that are highly bound to either plasma proteins or erythrocytes are unlikely to passively diffuse into milk (7). Overall, the amount of protein-bound chemical that enters milk is of little concern because the protein compartment in the blood is far greater than that in breast milk.

Other factors that affect the presence of a chemical in breast milk are its degree of biotransformation and its elimination rate. Frequently, the biotransformation processes, Phase I and Phase II, produce a metabolite that is more water soluble than the parent chemical and is readily eliminated through the kidney into the urine; hence, the chemical is not available for incorporation into breast milk. Less frequently, the metabolite is sustained in the blood and tissues, including breast milk, and is more readily measured than the original chemical (e.g., aldrin is metabolized to dieldrin and DDT is metabolized to DDE). With regard to the elimination rate, chemicals with a slow elimination rate have a long half-life, which allows for more time in the body and hence more time for bioaccumulation in breast milk. Many halogenated compounds, including the organochlorine insecticides (e.g., DDT and cyclodienes), polychlorinated biphenyls (PCBs), polybrominated biphenyls

(PBBs), polychlorinated dibenzo-*p*-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs), have long biological half-lives and are persistent in the environment and in humans because of their resistance to oxidative degradation and metabolism. The number of halogen atoms and the position of the halogen atoms on the molecule modulate these enzymatic processes (8). For example, the reported biological half-life for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is 7.2 years, whereas the reported estimates for other dioxins are 3.7 years for 1,2,3,4,6,7,8-heptachlorodibenzo-*p*-dioxin and 15.7 years for 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin (9).

For many of these long-lived environmental chemicals in lactating women, breast milk may be a major route of their elimination (10). Lipid-soluble chemicals are transported from adipose tissue stores to the lipids in breast milk and then eliminated from the body during breast-feeding. Thus, during a single lactation period or after several children and lactation periods, the concentration of a persistent chemical tends to decrease, assuming only background exposures to that chemical. This process of decreasing levels of chemicals during the breast-feeding period is known as “deuration” (11). For example, the half-life of total PCBs and DDT in human milk has been estimated to be approximately 6 months (12).

### The Role of Breast Milk in Biological Monitoring Programs

In the body, lipophilic chemicals are stored and equilibrated in tissue compartments with high fat content (e.g., breast milk and adipose tissue). In actuality, adipose tissue, breast milk, and blood or its components have been used for biological monitoring for assessing human exposure to lipophilic chemicals. Biological monitoring of these chemicals has several advantages over environmental monitoring because the former measures the internal dose rather than the exposed dose, and accounts for exposure from all sources (residential and work), all environmental pathways (through air, water, food, soil, surfaces), and all routes of absorption (ingestion, dermal, inhalation). However, biological monitoring may or may not yield information about specific sources, routes, and pathways that are important for risk management purposes. The most common reason for large-scale national biomonitoring programs is to monitor a population's exposure to chemicals, such as organochlorine insecticides, industrial chemicals and by-products (PCBs, PCDDs, PCDFs, lead), and solvents, and to determine whether a population's exposure to these chemicals is changing over time. The consequence of such information assists in

the determination of directed efforts in research, regulation, and policy design to improve public health care and safety.

In the United States, breast milk has not recently been used to a large degree in biomonitoring programs. The largest biomonitoring studies involving breast milk in the United States were conducted at Colorado State University. The first, conducted from 1974 to 1976, comprised 1,436 nursing women in hospitals; their milk samples were analyzed for selected chlorinated hydrocarbon insecticides and later for PCBs (13). The second national study, conducted from 1977 through 1983 by the same laboratory, comprised a total of 1,842 milk samples, which also were collected from women residing throughout the United States and were analyzed for the same organochlorine insecticides and PCBs (13). Also, in 1980 the U.S. Environmental Protection Agency (EPA) reported qualitative and semiquantitative data on levels of volatile organic compounds and semivolatile organic compounds in milk samples collected from lactating women in five U.S. cities (14). Despite these earlier initiatives, milk has not been routinely monitored in recent national surveys, such as the National Human Adipose Tissue Survey (15), which analyzed adipose tissue, and the National Health and Nutrition Examination Surveys (16) and the piloted National Human Exposure Assessment Survey (17), which both monitored blood and urine. However, breast milk has been more widely used in biomonitoring programs in Europe and Canada (18). For example, Norén and Meironyte (19) in Sweden and Fürst et al. (20) in Germany reported that the milk levels of PCDDs, PCDFs, and PCBs decreased dramatically from the early 1970s to the late 1990s. In contrast, Norén and Meironyte (19) reported a dramatic increase in milk levels of selected polybrominated diphenylethers (PBDE) congeners over this period. The World Health Organization (WHO) European Centre for Environment and Health is conducting its third field study, which is designed to assess levels and changes in levels of PCDDs, PCDFs, and selected PCBs in breast milk in countries worldwide (21).

Breast milk is a convenient specimen for biomonitoring programs because relatively large volumes (50–100 mL) can be collected noninvasively. This makes it a suitable matrix to be sampled in a large and easily identified population, albeit a selected population of women of reproductive age who are lactating. Because only this specific sample demography can be used, the use of mother's milk in a probability-based survey, the results of which are intended to be extracted to the general population, is questionable. Nevertheless, exposure in this segment of the

population obviously is important to monitor, and milk is important to monitor for contaminants because breast milk is a human food and is the major route of exposure to these contaminants by the breast-feeding newborn.

Another reason to monitor breast milk is that it reflects the maternal total body burden for lipophilic chemicals. Furthermore, concentrations of lipophilic chemicals in breast milk indicate the levels of these chemicals in the mother's fat stores during pregnancy and, consequently, provide a dosimeter of prenatal exposure to these chemicals. If used for this purpose, the milk should be collected in a narrow window of time as soon postpartum as possible to reduce the effects of depuration. However, any fat-containing matrix could be sampled from the mother and used for this dosimeter because lipophilic compounds partition within the body primarily on the basis of the fat content of the tissue. The average fat content of mature breast milk is about 4%. In contrast, serum contains 0.5–0.6% lipids and adipose tissue may range in lipids from 65% to > 90%, depending on the location in the body from where it is taken and the method used for procuring it (e.g., surgical, needle biopsy, or liposuction). The lipid-partitioning effect was demonstrated in a study of a population exposed to varying levels of TCDD (22). Patterson et al. (22) reported that, on average, levels of TCDD were 158 times higher in adipose tissue than in serum, but when each matrix was adjusted for its lipid content, the levels of TCDD were comparable in each matrix. Thus, tissue perfusion and the lipid partition coefficient [or bioconcentration factor (23)] play important roles in the distribution of chemicals in the body. In the case of PBBs, their concentrations in breast milk were reported to be 0.7–0.9 times that of adipose tissue when results from both were reported on a lipid-adjusted basis; in the same study the lipid-adjusted adipose tissue and breast milk concentrations were 107–119 times that of plasma, when results from the latter matrix were reported on a whole weight basis (24).

However, the level of fat and the level of environmental chemicals in breast milk require consideration of several factors beyond the degree and duration of exposure, the effects of depuration, and the time of sampling during lactation. These include characteristics of the mother. Maternal features that affect these levels include the health of the mother during pregnancy and during the lactation period, presence and levels of other xenobiotics (including environmental chemicals and pharmaceutical agents) that may alter metabolism, change in body mass index during pregnancy and lactation, diet,

other factors that may mobilize fat, parity and length of previous lactation, number of children being breast-fed at one time, maternal age, and maternal body mass index. Also of importance is the variation of the fat content during lactogenesis and during the course of feeding (25–27). We have already mentioned that the fat content during lactogenesis tends to increase from about 2.9% during the first few days and then stabilizes at around 4% after 2–6 weeks. During the actual time of breast-feeding, the foremilk can have a fat content of about 1% and the hindmilk can have a fat content of up to 12%; levels of fat can also differ between the two breasts. Also, if an infant empties the milk content of one breast, the foremilk from the second breast is generally higher in fat content than the foremilk was from the first breast. For monitoring surveys, breast milk should be collected once the fat content has stabilized; the protocol of WHO-Europe calls for sampling 2 weeks to 2 months after delivery (21); however, more studies need to be conducted to determine whether the milk is comparable during this 8-week period.

### The Role of Breast Milk Monitoring in Epidemiologic Studies

In addition to monitoring breast milk purely for exposure assessment purposes, researchers have analyzed breast milk to determine the relation between concentrations of environmental chemicals and adverse effects in humans; thus, the determination of potential health consequences of contaminated breast milk to the infants and their mothers is another important reason to conduct biological monitoring in breast milk. Several incidents have been reported of women being exposed to chemicals, then breast-feeding their children with contaminated milk, and the children having adverse effects, especially neurodevelopmental effects. These reported chemicals include PCBs (28–31), PCBs/PCDDs/PCDFs (32–34), PBBs (35), hexachlorobenzene (36), methylmercury (37,38), and DDT (25). Although transplacental exposure is generally believed to be more consequential to the health outcomes than exposure through breast milk, this has been debated (39,40). If these measurements in breast milk had not been conducted, such information about contributory effects on health outcome would not have been recognized. Nevertheless, because the significant nutritional and immunologic benefits from breast-feeding outweigh the limited adverse health effects from the presence of environmental chemicals in breast milk, the American Academy of Pediatrics continues to recommend breast-feeding in most circumstances (41).

Concerns have been raised about the levels of environmental chemicals in breast milk and other human tissues and effects on the mother. Women with a higher DDE concentration in their breast milk have a shorter duration of lactation than do those with a lower concentration (42,43). Another concern is for the occurrence of breast cancer with exposure to organochlorine chemicals (e.g., DDE, DDT, PCBs). This association has not been consistently demonstrated (44).

### Analytes Monitored in Breast Milk

Before describing the analytic considerations for analyzing breast milk for environmental chemicals, we need to list the analytes of interest.

**Organohalogenes.** The first class of chemicals generally discussed in breast milk monitoring programs is the organohalogenes, which include the organochlorine insecticides, PCDDs, PCDFs, PCBs, PBBs, and PBDEs. On the basis of analytic chemistry methods, these chemicals are considered to be semivolatile and are generally measured by gas chromatographic methods after an extraction process.

**Volatile organic compounds.** In the U.S. EPA's analyses of breast milk collected from five U. S. cities, 26 halogenated hydrocarbons, 17 aldehydes, 20 ketones, 11 alcohols, 2 acids, 3 ethers, 1 epoxide, 14 furans, 26 other oxygenated compounds, 4 sulfur-containing compounds, 7 nitrogen-containing compounds, 13 alkanes, 12 alkenes, 7 alkynes, 11 cyclic hydrocarbons, and 15 aromatic compounds were found, including significant amounts of hexanal, limonene, and dichlorobenzene (14). These chemicals are generally measured by gas chromatographic methods following a purging or headspace sampling process.

**Metals.** Heavy metals, including lead, mercury, and cadmium, are seldom monitored in breast milk; one reason is that their levels are only about 20% of their levels in maternal blood. These metals can be present either in their inorganic forms or as organometallics. They are generally measured by atomic absorption spectroscopy (AAS), inductively coupled argon plasma spectroscopy, or mass spectrometry (MS), often following a digestion process.

**Other chemicals.** As in most cases, the "other chemical" category contains a variety of chemicals ranging from contemporary pesticides, polycyclic aromatic hydrocarbons, nicotine, ethanol, phthalates, musk xylenes, and phytoestrogens, such as genestein. These chemicals are generally determined by either gas chromatography (GC) or high performance liquid chromatography methods (HPLC).

## The Analytic Method

The major methodologic goals of analyzing breast milk for environmental chemicals are the same as for analyzing any other biological sample or, for that matter, any environmental sample for those environmental chemicals. The goals of the method are built around specificity, sensitivity, robustness, ruggedness, accuracy, precision, ability to measure multiple analytes, and high throughput. However, the analytic approach for analyzing milk for environmental chemicals must take into account the fat content of milk. This leads to some general (e.g., specimen collection, preparation) and specific (e.g., congeners) considerations for measuring environmental chemicals in breast milk.

## Specimen Collection

The sample must be collected under a well-designed protocol; this includes signed consent forms and approval by an Office for Human Research Protection-approved Institutional Review Board. Key issues of breast milk collection are the adherence to the protocol, administration of a well-designed questionnaire, accurate labeling of all containers, avoidance of contamination, and the adequacy of the specimen. The breast and hands should be clean, yet soap should be avoided as much as possible. The use of creams or ointments on the nipples should be used outside the sampling time for the analysis, but if this is not possible because of tenderness, the breasts should be washed thoroughly and rinsed with copious amounts of water before sampling. The milk can be expressed either manually or by a breast pump, but if a pump is used, it must be free of contamination. Some groups recommend collecting the milk specimen from one breast using an electric pump while the baby feeds on the other breast in order to take advantage of the let-down reflex. The collecting bottle must be provided free of contamination and should be washed, rinsed with water, and rinsed with acetone before being given to the mother. If the analysis calls for inorganic elements, the collecting bottle is generally acid rinsed. At least 50 mL of milk should be collected in a wide-necked glass bottle; if the milk is expressed manually, avoid contact between the breast and the jar. In general, glass; Teflon; certain plastics, such as polyethylene; and aluminum foil (if only organic chemicals are measured) are suitable to come in direct contact with the specimen. Certain plastics, such as polyvinyl chloride, and metal containers are to be avoided because their constituents can interfere with certain detectors interfaced with the analytic instrument. Aluminum foil (dull side down) or Teflon is generally used as liner material for the lid.

## Specimen Storage and Transport

If the breast milk is collected several times over a 72-hr period, then the milk in the collection container should be stored in the home freezer or in a home refrigerator. Tablets of potassium dichromate, a preservative, may be added if necessary. If volatile organic chemicals are not intended to be measured, the milk specimen should be warmed to 38°C and inverted several times to mix the cream layer, and then divided into aliquots into several bottles to minimize the effects of freeze-thaw cycles. These bottles should have labels that will remain intact and readable throughout transport and storage (45). The bottles containing the samples should be placed in a thermoinsulated box with dry ice; the bottles should not contact each other or the dry ice. The laboratory staff should be notified of the shipping of the samples and given all information about the shipping. When the samples arrive, the laboratory staff should remove the bottles from the shipping box; note the condition of the labels, the bottles, and the milk; and relay this information back to the shipper. For the measurement of most analytes, the samples can be stored indefinitely at -70°C.

## General Analytic Approach

Several methods exist for measuring environmental chemicals in breast milk. Of course, as mentioned above, the specific method depends on the analytes of interest; therefore, specific methods are not practical to discuss without listing the specific chemicals or at least classes of chemicals. We classify the environmental chemicals as either inorganic chemicals (including organometallic compounds) or organic compounds, which are further divided into volatile or semivolatile classes. The measurement process of the chemicals of concern consists of four primary steps: lipid determination; sample preparation; instrumental analysis, which usually involves a chromatographic step; and data analysis and evaluation. Before the measurement process, the samples to be analyzed in an analytic run or batch should be removed from the freezer, checked for proper labeling, brought to 38°C (unless they are to be analyzed for volatile chemicals), and mixed by gentle inversion. The number of samples that can be analyzed by an analyst or a team of analysts in 1 day generally determines the number of samples included in an analytic run. Into this batch are added quality control samples that consist of milk that has been fortified with the analytes and/or milk that has not been fortified, as well as a solvent blank, which is often purified water that is analyzed in the same manner as the milk samples. The quality control samples can consist of bench or internal quality control

samples and external or blind quality control samples. Generally, both of these samples are prepared in a bulk manner and then the milk is transferred by pipette to the container so that the quality control samples are identical in appearance to the unknown samples. In our study, both the bench and the blind quality control samples have been analyzed by the laboratory numerous times, and the mean values and control limits have been determined for the analyses. Laboratory personnel know the positions of the bench quality control sample and of course the quality of the blank in the analytic run, but they do not know the position of the blind quality control sample. We also recommend analysis of standard or certified reference materials, if they are available, for the particular analytes in breast milk.

## Sample Preparation and Lipid Determination

**Semivolatile organic compounds.** The purposes of the sample preparation step and lipid determination step are to extract the fat components and the analytes of interest from the remainder of the milk sample, to determine the fat content of the milk sample, and to further prepare the extract for instrumental analysis. The lipid content may be measured in the entire amount of sample or in an aliquot of the milk specimen. In either case, the homogenized milk sample is treated with a denaturing agent such as ethanol, sodium oxalate, or formic acid, and the lipids are extracted into an organic solvent. The extract is sometimes passed through a column containing a drying agent such as sodium sulfate to remove traces of water. The solvent is concentrated to dryness and the lipid weight is determined gravimetrically. The percentage of the lipid content of the specimen is determined especially when lipophilic compounds such as PCBs and organochlorine insecticides are measured because, most frequently, the concentrations of the analytes of interest are reported on a lipid-adjusted basis as well as on a whole-weight basis. The lipid-adjusted basis normalizes the concentrations of the lipophilic environmental chemicals to the different lipid contents of the milk, both within a mother and among mothers. The lipid-adjusted concentrations of lipophilic compounds are used for population estimates of these chemicals in the mothers and for defining infant intake of these chemicals. These chemicals must be accurately and precisely measured on a whole-weight basis to adjust their concentrations to a lipid basis. This lipid determination step, however, is critical, and because of the potential for incomplete extraction, incomplete concentration of the sample, and weighing errors, the overall error is often more associated with the

measurement of the lipids than with the measurement of the analytes. This was demonstrated in the first WHO-sponsored interlaboratory study for measuring PCBs, PCDFs, and PCDDs in breast milk (46). In addition to the liquid/liquid extraction method, milk samples have also been prepared for further analysis by using lyophilization or freeze drying of the milk.

The goal of the sample preparation step is to prepare the sample for instrumental analysis while maintaining the high recovery of the analytes but separating them from the lipids and from other coextracted potential interferants. The method used for the sample preparation step (sometimes called the “cleanup step”) is determined by the physical and chemical nature of the analytes and instrumentation available to the laboratory. This concept sounds straightforward but can often tax the ability of analytic chemists. For example, as we mentioned, human milk is generally 3–4% lipids, but the analytes of interest, such as PCDD and PCDF congeners, PCB congeners, and organochlorine insecticides, are generally present at parts-per-quadrillion, parts-per-billion, and parts-per-billion concentration levels, respectively. Thus, the method needs to extract the lipids and the analytes and separate all of these components from other components of the milk sample with high efficiency and specificity. To carry this one step further, the PCDDs, PCDFs, and coplanar PCBs must also be separated from the “regular” PCBs and organochlorine insecticides before instrumental analysis. The specificity, sensitivity, precision, accuracy, “ruggedness,” and robustness of the analytic method are highly related to the ability of this step to meet its goal.

Some methods used for cleanup for the measurement of semivolatile analytes include column chromatography, thin-layer chromatography, sweep codistillation, and gel permeation chromatography. More recently, chromatographic techniques such as solid phase extraction (SPE) have been used in the cleanup of many semivolatile chemicals, including pesticides and organochlorine compounds. A variety of sorbents are commercially available for solid phase extraction (SPE). These sorbents can be used in combination with the specific properties of the analytes to selectively isolate the compounds of interest from other components of the sample. SPE can be used in two different modes: the first is to initially retain the analytes on the column packing, thereby allowing potential interferants to pass through the column, and then elute the analytes with the prescribed eluent; the second is to retain the interfering substances, thereby allowing the analytes to pass initially through the column. If the second mode is used, typically an

additional preparation step is employed to isolate the components from the matrix. Another technique, which has the potential for extracting lipophilic compounds directly from milk, is stir bar sorptive extraction.

**Volatile organic compounds.** To analyze breast milk for volatile analytes, the sample preparation step is based on volatilization of the analyte, which can include a process based on volatilization of the analyte into the headspace above the sample or by a similar process followed by a purge of the headspace by an inert gas, then trapping of the analytes onto an adsorbent. The adsorbent is reheated and the analytes are released as part of the instrumental analysis process. A separate aliquot of the milk sample is generally used for lipid determination.

**Inorganic chemicals.** To analyze breast milk for metals, the sample preparation step is generally based on a digestion of the proteins in the milk, thereby releasing the metal to be measured by an instrumental analysis process. Lipid content may or may not be determined because the metals are generally not partitioned into the lipids.

### Instrumental Analysis and Quantification

Once the specimen has been prepared for instrumental analysis, the analytes of interest can be further separated from each other and from remaining potential interferants by high-resolution chromatographic techniques such as capillary column GC or HPLC. The method is selected depending on the physical and chemical properties of the chemical (e.g., volatility, thermal stability). Following separation, the analytes are detected using a variety of analytic instruments, including mass spectrometers, although historically halogenated, semivolatile organic compounds have been detected by electron capture detectors. Once detected, these particular compounds are quantified by two primary methods, the external standard method and the internal standard method. The external standard method is generally the less accurate of these two quantitative techniques. It relies on a standard curve consisting of the detector responses of the analyte ( $y$ -axis) versus the varying concentrations of the analyte in standard solutions ( $x$ -axis) and generally makes no amends for losses of the analyte during the analysis of each unknown; however, recovery losses can be estimated by using one of two approaches. The first approach is to incorporate a surrogate compound in the milk sample and monitor its recovery throughout the analytic process; for example, the analyst may add a PCB congener that is generally not detected in human milk to the milk sample and monitor its recovery. The recovery of the analytes of interest can be

inferred from the recovery of the added PCB, although no adjustments for losses are made when reporting the concentration levels of the analytes. The second approach is to spike a milk pool with known amounts of the analytes of interest, analyze the milk pool many times, and calculate and average the recovery of the analytes over time. The analyst then assumes that during the analyses of the actual samples, the analytes are recovered to a similar degree, but again the analyst does not take this into account during the reporting of the concentration levels of the analytes.

The internal standard method involves the addition of one or more compounds (the internal standards) that ideally are recovered to the same degree (but at least to a consistent relative degree) as the analytes during the analytic method; this relative behavior is determined by previous analyses of milk pools spiked with the analytes and the internal standards. The internal standards should be added and equilibrated into the sample before the extraction step. Therefore, any losses of the analytes during the analytic method are accompanied by similar losses (or at least defined losses) of the internal standards. The quantitative result for a given analyte in a milk sample is calculated from a standard curve consisting of the ratio of the detector responses of varying concentrations of that analyte divided by the detector responses for a constant concentration of its internal standard ( $y$ -axis) versus the varying concentrations of the analyte in standard solutions ( $x$ -axis).

The isotope dilution MS technique for quantification is essentially the same as the internal standard quantification method with two exceptions. The first exception is that the internal standard is actually the same chemical (differing only in isotopes) as the analyte; therefore, the stable isotopically labeled internal standard should chemically and physically mimic the analyte, and any loss of the analyte during the analytic process should be accompanied by a similar loss of its internal standard. Its use allows for a complete accounting of any loss of analyte and thus “adjusts” the recovery to 100%; therefore, as with the internal standard method, no recovery calculations have to be made. The concentration level of the analyte is calculated in a similar manner as described for the internal standard method. The second exception is that because the analyte and internal standard are the same chemical (differing only in isotopes), their primary difference is only in mass, which means that the instrumental detector must be able to distinguish the analyte from the internal standard on the basis of mass (i.e., a mass spectrometer must be used). For increased sensitivity that is often required in milk analysis, the mass spectrometer generally monitors only selected ions. The use of the isotope

dilution quantification technique and the combination of specificity and sensitivity of mass spectrometry, especially when used in the selective ion monitoring mode, can provide the basis for “definitive” methods in analytic chemistry. The only pertinent weakness, besides the expense of instrumentation and the labeled standards, is the inability to qualitatively distinguish among certain isomers that have similar chromatographic properties and mass spectral fragmentation patterns (i.e., structural isomers that coelute); in such cases, Fourier-transform infrared spectroscopy can be used, even though it does not have the same inherent sensitivity as MS in the selected ion monitoring mode. However, with the advent of many more widely varying chromatographic columns, including those based on chirality, this weakness is seldom relevant. From a quantitative viewpoint, the major sources of error in IDMS are in the purity of the native (unlabeled) standards and sometimes the labeled standard, and the inaccurate addition of the prescribed amount of the solution containing the labeled standard. A fourth method of quantification, the spiked addition method, is seldom used because repeated analyses are required for each sample. The first analysis involves the analysis of the sample without the addition of any native standard to the sample, but each successive analysis involves the addition of increasing amounts of labeled standard. A calibration plot is constructed from the areas of the spiked samples, and the  $x$ -intercept is representative of the concentration of the analyte in the milk sample.

The mass spectrometers used for measuring environmental chemicals in milk can range in price from the relatively inexpensive mass selective detectors (around \$100,000 U.S.) to tandem mass spectrometers (around \$300,000 U.S.) to high-resolution mass spectrometers with various designs and configurations (range from \$500,000 to \$1,000,000 U.S.). With proper laboratory technique, the use of pure standards, and IDMS, the instrumental analysis step seldom leads to imprecise and/or inaccurate measurements.

### Data Analysis and Evaluation

Most laboratories use computers to better track the status of samples from their delivery into the laboratory to reporting of the data, to control the instruments for analyzing the sample, and to calculate the results. This generally results in fewer laboratory errors. Nonetheless, laboratory personnel must be aware of the potential for errors, must properly set up each of the computers, and must check the output of each step. They should help determine which data output parameters are necessary and their specifications for determining the quality of the data and for determining

whether the results for an individual sample and/or for the entire analytic run are “in control.” The determination of whether the entire analytic run is in control is based, to a large degree, on the results of the quality control samples. These quality control samples also evaluate the performance of the analytic method in a given laboratory over time. The determination of whether the results for a particular milk sample are in control is generally based on other factors relevant to the type of analyses. For example, for GC/MS methods, these quality control factors may include the retention times of the analytes and the internal standard; the degree of resolution of the analyte from other analytes or contaminants; the percent recovery of the internal standard; and, particularly if halogenated (chlorinated or brominated) compounds are being measured, the ion ratios of the halogen atoms (e.g., if the analyte contains one chlorine, the contribution of  $^{37}\text{Cl}$  should be about one-third that of  $^{35}\text{Cl}$ ). If compounds not containing halogen atoms are being measured, certain ion(s) can be used for quantification and another for confirmation in much the same manner.

Of particular concern in trace analysis is how to report and statistically treat concentration levels that are below the limit of detection (LOD). The LOD is defined by the lowest concentration of chemical that the analytic method can measure. It is determined by the measured value that differs in a statistically significant manner from having “zero” chemical in the specimen (47). The efficiency of the analytic method in preparing extracts free of potential interferants (but still recovering a high percentage of the analytes of interest) and the sensitivity of the instrumental system affect the LOD for the method. The LOD should be determined in each laboratory for each instrument (instrumental LOD) and for each method (method LOD); frequently, the method LOD is calculated for each and every sample analysis. When measurements are calculated to be  $< \text{LOD}$ , the concentrations are generally reported as “nondetectable” with the LOD given. However, for parametric statistics, a number must be assigned for each sample. To circumvent this problem, values ranging from the most “conservative” value of zero to one-half of the detection limit concentration, to the detection limit divided by  $\sqrt{2}$ , to the most “liberal” value—the detection limit itself (48)—have been used. More sophisticated modeling methods have also been used to estimate the concentration levels for nondetectable results (49). From a laboratory perspective, our general policy is to statistically use all values determined by MS that are at or above the LOD and that pass the quality control criteria (for the run; for the sample; and if the

analysis is for multiple analytes, then also for individual analytes within the sample). However, what about reporting concentrations when they are below the LOD? Two scenarios exist: For the first, all of the quality control criteria are again met except that the calculated concentration value is below the LOD; in this case, we report the calculated value with the caveat that the result is below the LOD—for statistical purposes in our epidemiologic studies, we believe that this is the most accurate value to use for that sample. For the second scenario, the quality control criteria for the analytic run are met but no signal for a particular analyte is discernible; those results are reported as below the LOD, and no number above zero can be assigned to them. Other researchers treat both scenarios as the same and report all results below the LOD as nondetectable and then assign, for statistical purposes, a number as described above (e.g., one-half the LOD). Because our analytic methods have become much more sensitive, concentrations of targeted chemicals in the breast milk from exposed populations, as well as from the general population, are seldom calculated as being below the LOD; however, we are still faced with this problem. As shown by many researchers, our exposure to many environmental chemicals is decreasing (fortunately), and their milk concentration levels may not continue to be sufficient for detectable measurements. Another scenario that can lead to nondetectable results is that we often measure multiple analytes in an analytic run, and the more analytes that we measure in an analytic run, the lower the sensitivity for the measurement process of all of the analytes, either because of instrumental reasons or for recovery reasons. Therefore, the use of multianalyte methods has many advantages, but generally they have higher LODs than single analyte methods.

One approach to help ensure the availability of an adequate volume of milk for measuring low levels of environmental chemicals is to combine individual samples of similar demographic or presumed-exposure characteristics into a single sample to prepare a “pooled sample.” This practice is widely used to decrease the number of samples to be analyzed and consequently it allows for a larger number of mothers to be represented in the survey; in addition, it saves laboratory resources. Another benefit of pooling specimens is the use of small volume specimens that would otherwise be excluded from analysis. However, some issues should be considered when specimens are pooled: the loss of direct association with the donors; the inability to perform statistical analysis between pooled populations because of the loss of variability within the pooled population; the reporting of estimated instead of

actual measurements of the analyte in the population; and the disproportionate weighting of the measurements because of dilution. The creation of subgroups within each pool can establish a variance for the pool to allow for comparison between pools. Additional consideration needs to be given for the variability in lipid content relative to time of breast-feeding among the donors before the specimens are pooled. Therefore, whether the volume of each sample to be pooled should be based on a given volume per sample or a given amount of lipids per sample becomes questionable. Although the latter method may be preferred, it is seldom used because it requires the additional measurement of the lipid content of the samples before pooling.

### Analytic Issues with Specific Chemicals

**PCDDs, PCDFs, and certain PCBs.** The PCDDs, PCDFs, and certain PCB congeners represent classes of many compounds that exhibit similar mechanisms of toxic actions. There are 75 congeners of PCDDs, 135 congeners of PCDFs, and 209 PCB congeners. All of these congeners could potentially be dispersed into the environment. However, not all of the congeners bioaccumulate in the food chain and hence are not found in the fatty stores in humans. For example, we generally consider that only 7 of the PCDDs and 10 of the PCDFs are stored in the fatty tissues of the human body. That is the good news. The bad news is that these 17 are the most toxic members of these two classes of chemicals. The most toxic congeners have four or more chlorine atoms substituted (for hydrogen) on the aromatic rings, and all four of the lateral positions (the 2, 3, 7, and 8 positions) must be substituted with a chlorine atom. Because of their dioxin-like activity, 12 PCBs are generally reported along with the PCDDs and PCDFs; 4 of these PCBs (the coplanar PCBs or non-*ortho*-substituted PCBs) have no chlorine substitution in either of the four *ortho*-positions (the 2, 2', 6, or 6'-positions) and 8 of these PCBs (the mono-*ortho*-substituted PCBs) have only one of these four positions substituted with a chlorine. Therefore, approximately 30 different chemicals are reported as dioxin-like chemicals in humans. Because so many congeners need to be monitored for dioxin-like activity, methods to facilitate the interpretation and reporting of the data (as well as to establish regulatory limits) were adopted. In short, the reported value for dioxin-like chemicals is frequently condensed to one value—the toxic equivalency (50).

The toxic equivalency is a weighting factor derived by multiplying the concentration of each of approximately 30 individual dioxin-like chemicals by their respective

toxic equivalency factor and then summing these values. The toxic equivalency factor is a relative factor based on ability of each chemical (relative to the most toxic congener, TCDD) to induce cytochrome P450 1A1 and its affinity for the aryl hydrocarbon (Ah) receptor. The toxic equivalency approach allows for a meaningful comparison of the toxicologic contribution of each dioxin-like compound and a comparison of the concentration of each of these compounds within a biological or environmental sample and between samples. Some of the limitations to this approach include the nonadditive antagonistic effect of various chemical mixtures (51) and mechanisms of toxicity not appreciated by either the Ah receptor or cytochrome P450 activity (52).

Another approach that has been developed recently for measuring dioxin-like total equivalents is the chemical-activated luciferase gene expression (CALUX) bioassay (53). This method, based on the Ah receptor agonistic activity of these chemicals, is less expensive and has higher throughput than methods based on GC/MS. However, the method still requires an extraction, lipid determination, and cleanup of the milk sample. Furthermore, the final result is only a toxic equivalent and thus gives no information about the dioxin pattern of exposure, which yields information about the exposure scenario, including the source. Also, frequently this method overestimates the concentration of PCDDs, PCDFs, and dioxin-like PCBs because other components in the breast milk may possess Ah agonistic activity. Nonetheless, this method is suitable for screening of samples to estimate dioxin levels and for prioritizing those samples for dioxin analysis by GC/MS.

Another area of interest in measuring chemical classes with many congeners, such as PCBs, is the interpretation of residue data. The toxic mechanism of action for most of the PCB congeners is different from that of dioxins; in fact, the 209 PCB congeners can act through a variety of toxic mechanisms. So, from a toxicologic standpoint it is important to be able to separate and quantify each of the congeners that appear in breast milk. Of course, not all of the congeners are present in any specimen, environmental or biological; the number of congeners identified depends not only on the exposure scenario but also on the analytic method. Over the years, as technology and methods have improved, so has the ability to chromatographically resolve more compounds and to measure them with improved sensitivity. Thus, PCBs are now frequently reported as individual congeners, although the individual congeners themselves are frequently summed to give total PCBs; this is done to allow for direct comparison with historical data and

for ease of correlation with health end points, although this approach may be flawed for both uses. Historically, total PCBs were calculated from comparison to the commercial material that chromatographically was most similar to the chromatographic pattern of the extract from the biological sample; however, little or no information was available for individual congeners. Methods were developed for calculating total PCBs from these chromatograms. However, these methods of interpretation led to an over estimation of the actual measurement of the chemical class compared with analytic methods that can resolve all the congeners (54). Thus, the comparison of data among laboratories with different abilities to resolve many congeners can be problematic. Data sets can be best compared from laboratories that can identify and quantify a similar number of congeners using similar techniques.

**Heavy metals.** Heavy metals appear in milk at smaller concentrations than the lipid-soluble chemicals and are about 20% of the level found in blood from the same person. This is attributed to their low lipid solubility and high binding to erythrocytes. The amount of heavy metal exposure to the infant from breast milk appears to be low in comparison with other sources; further information is needed about the pharmacokinetics of the heavy metals (especially lead) as they go from mother to infant. An area of concern regarding the analysis of heavy metals is the identification of the various species of metals (especially for mercury and arsenic). This is important because different species of metals have varying toxic effects. For example, methylmercury, which binds to sulfhydryl groups, is a central nervous system toxicant and inorganic mercury is a nephrotoxicant. Thus, the need to be able to measure individual species of the heavy metals becomes apparent. AAS is commonly used to detect heavy metals, which can be speciated by using selective specimen preparation methods. The LOD with AAS can be decreased with flameless (e.g., electrothermal) atomizers.

### Future Trends

The assessment of exposure to environmental chemicals in breast milk from the general population needs to pursue the measurement of environmental chemicals that have not been well characterized and have potential adverse health effects (e.g., pesticides, such as organophosphates; xenoestrogens; brominated aromatic hydrocarbons; solvents; and polycyclic aromatic hydrocarbons). In addition, breast milk monitoring needs to be increased in populations that may be unduly exposed to certain chemicals that are easily measured in breast milk. As mentioned previously, breast milk is a

unique biomonitoring matrix in that it is also a human food, and although the exposure lasts for a relatively short and limited time, the amount of chemical intake on a daily basis may far exceed the public health criteria, which are generally based on an exposure period of 70 years. Public health officials need to recognize this. In addition, chemists need to measure these chemicals in an accurate and precise manner. Therefore, the chemist must be kept abreast of laboratory criteria in order to help ensure the quality of the data. An increasing number of laboratories will have to demonstrate quality through such programs as the Clinical Laboratory Improvement Amendment of 1988 (55) and interlaboratory studies. The chemists are responsible for providing accurate and precise analytic data for assessing exposure so that the relationship between exposure and adverse health outcomes can be made most accurately; this in turn should lead to improvements in legislation and regulation that protect people but not to an unwarranted degree.

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