

Review

Hyphenated Techniques and NMR Methods for Possible Organochlorinated Pesticides Occurrence in Human and Animal Milk

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Abstract: Although not expected to be used due to restrictions raised on their usage, Persisted Organic Pollutants (POP) such as organochlorinated pesticides (OCPs) can be found in several matrices, even nowadays. The lack of biodegradation and, furthermore, their persistence in the environment result in the possible occurrence of these lipophilic toxins in several matrices, from environmental samples and foods to human milk. The current review focuses on the usage of hyphenated techniques for the determination and monitoring of OCPs in several matrices, such as milk—both animal and human milk. The lipid matrix of milk and dairy products favors the possible bioaccumulation of the above pollutants, and the complex matrix of the dairy products is a challenge for method development. Additionally, spectroscopic methods—mainly Nuclear Magnetic Resonance (NMR)-based metabolomics—for biomonitoring of OCPs persistence, bioaccumulation, and effect of possible exposure, along with NMR usage in several methods developed, are also presented and discussed. Finally, we introduce and present the metabolomic approach for OCPs and other POPs in lipid matrices.

Keywords: persisted organic pollutants; organochlorinated pesticides; extraction; metabolomics; NMR; chromatography; sample preparation



Citation: Thanou, E.D.; Tsiafoulis, C.G. Hyphenated Techniques and NMR Methods for Possible Organochlorinated Pesticides Occurrence in Human and Animal Milk. *Separations* **2024**, *11*, 282. <https://doi.org/10.3390/separations11100282>

Academic Editor: Alberto Barbiroli

Received: 9 September 2024

Revised: 22 September 2024

Accepted: 25 September 2024

Published: 29 September 2024



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1. Introduction

Human exposure to pesticides happens mainly through the environment, work, and diet. Exposure to pesticides is one of the most dangerous factors because people get in frequent contact with large doses of pesticides. Once individuals are exposed by one of the above routes, pesticides enter the human body, causing acute and even chronic toxicological effects [1]. The nature and severity of this effect depend on the individual chemical properties, dose, duration, and way of exposure, as mentioned above. Human milk (HM) is the most efficient source of nutrients such as proteins, carbohydrates, fats, vitamins, and immune factors and provides important components for the development of infants [2]. On the other hand, possible hydrophobic pesticide occurrence, such as organochlorinated, can be monitored in such a rich in lipids matrix. In fact, human milk is considered an ideal substrate for biomonitoring of possible exposure to lipophilic pollutants [3]. Moreover, POPs bioaccumulate in human breast milk [4].

DDT belongs to the group of molecules that have been identified as endocrine disrupting chemicals (EDCs) altering the “hormonal and homeostatic systems that enable the organism to communicate with and respond to its environment” [5].

Exposure and accumulation of xenobiotics through several paths such as soil, air, diet, metabolism, and time range results in the pollution rate of humans (Figure 1) [6].

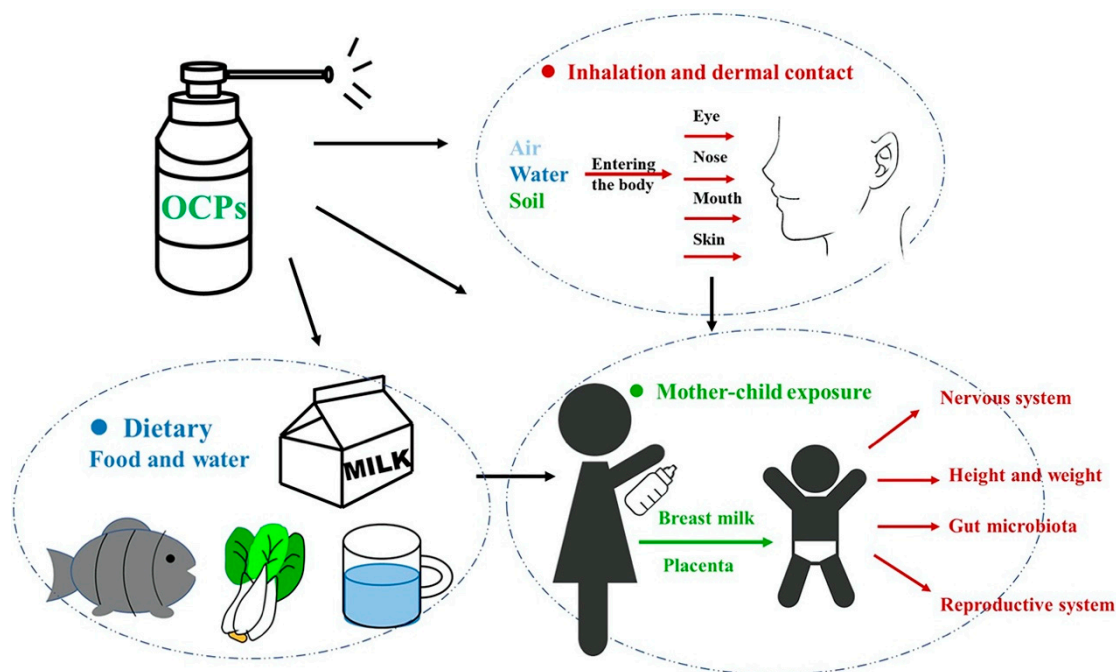


Figure 1. Direct or indirect exposure to OCPs (adapted from [6]).

The utmost possible existence of endocrine-disrupting Organochlorinated Pesticides (OCPs) in HM [7,8] raises major concern due to the hazardous health risk that they pose to infants [6,9], affecting also the embryo—for instance, the birth size [10]. Prenatal exposure, e.g., through placenta concentrations of xenoestrogenic OCPs [11], is of major concern along with maternals' exposure to OCP residues. The possible maternals' exposure to OCPs has an impact on the infants—though their milk—and several counties have been screening for possible organochloride occurrence in human milk [12]. Utmost, the OCPs occurrence in plasma could possess a risk factor for three generations, grandmother-to-mother-to-daughter [13]. Following the above, HM is of major importance, and the possible occurrence of OCP residues—although of concern even from previous decades—has also been studied nowadays by several authors for a series of regions around the world.

A pesticide is defined as a substance or mixture of substances used to fight a parasite. Pesticides can be chemicals, biological organisms such as viruses or bacteria, or antimicrobial or disinfectant substances. The OCPs are one of the categories of pesticides available. More specifically, they are substances that have a chemical bond between chlorine and carbon [14].

Pesticides are also used for the control of disease carriers such as malaria and contribute to their prevention. Despite their importance, pesticides are considered chemical pollutants and pose a serious threat for human life and the environment. OCPs are more likely to be chosen due to their durability, high toxicity to parasites, and their low price. These substances are also very resistant to heat, radiation, and biodegradation, which enables them to remain insoluble and act without changing their composition in water and soil. The above creates great concern to scientists as they pose a real danger to wildlife. Also, due to their lipophilicity, OCPs can accumulate in foods that are rich in lipids, among them dairy products. The latter, along with the fish, are the main source of human exposure [15].

OCPs are usually divided into five major groups [16], which include dichloro-diphenyl-trichloroethane (DDT) and its analogues, hexachlorocyclohexane (HCH), cyclodienes and similar compounds, toxaphene and related chemicals, and the caged structures mirex and chlordane (Kepone®).

Although most of the organochlorinated pesticides have been considered banned and eliminated for many years—the Stockholm Convention was implemented in May

2001—and in 2017, sixteen (16) chemicals were characterized as POP and distributed in A to C Annexes: for elimination or restriction of the usage and production for A and B or reduction of unintentional releases of chemicals in Annex C—the possible existence of OCP residues in both environmental samples or milk matrices level is monitored.

It is therefore necessary to have increasingly sensitive and reliable analytical techniques to ensure effective monitoring of OCP residues [17,18], ensuring thus the protection of public health and the study of possible environmental impact. These methods are so important for OCP monitoring because otherwise they can be very harmful to the human body. In milk samples, both animal milk (AM) and HM, the determination of analytes is considerably difficult as there are elevated concentrations of fats and proteins, which affect their determination through several analytical methods. For this reason, sample extraction can be time-consuming and tedious, involving several cleaning steps to discriminate the OCPs—which are to be measured—from the matrix in order to eliminate matrix effects.

The current Maximum Residue Levels (MRL) for a-Endosulfan, b-Endosulfan, Endosulfan-sulfate, DDE, c-HCH, Dieldrin, and DDT are 50, 50, 50, 40, 1, 6, and 40 $\mu\text{g Kg}^{-1}$, respectively [18]. European Union (EU) by COMMISSION REGULATION (EU) 2023/163 has set up a MRL for DDT (sum of p,p'-DDT, o,p'-DDT, p,p'-DDE, and p,p'-TDE (DDD) expressed as DDT) in milk at 0.04 mg kg^{-1} . ([19] EU 2023)

Nuclear Magnetic Resonance (NMR) methods can solve and enlighten both analytical chemistry information and several molecular interactions. For instance, novel techniques for OCP detection based on their interaction with bicycling amines have been reported using NMR for the interaction characterization [20]. Additionally, the sensing mechanism of methylammonium lead halide perovskite quantum dots with OCPs has been illustrated by ^1H NMR—among other techniques [21].

Moreover, through the *-omics* tools, NMR methods can provide novel insights in several scientific fields. For instance, in the field of food chemistry, the *-omic* approach has been applied in milk originality [22] and olive oil profiling [23]. Novel approaches, such as the metabolomic analysis for the toxicity effects of OCPs [24] and their effect on plasma [25] and on liver lipidosis in mice [26], have been recently reported. Additionally, NMR metabolomics have been used for the study of human exposure to OCPs—for instance, either with the examination of exposure to Persistent Organic Pollutants (POPs) through the analysis of human plasma [27] or through an exposome approach for urine metabolic signatures of environmental pollutants in pregnant women [28].

Herein, following the above, we report commonly used methods for OCPs determination in milk. Moreover, a novel approach for their determination and their hazardous effects through NMR spectroscopic methods.

2. Overview of the Recent Studies for OCP in Both HM and AM

As reported above, the possible OCPs' occurrence is of major concern. Several groups have studied it for a series of regions [2,9,29–36].

Additionally, recently, the first WHO/United Nations Environment Program (UNEP) survey on the current concentration of POPs—OPCs included—in human milk in Morocco has been published [37]. WHO/UNEP has also gone through a 19-year study (2000–2019) for POP in human milk; B. Hardebusch et al. [38] reported for analysis and Malisch [39] for the findings of organochlorine pesticides and industrial contaminants in human milk.

Souza et al. determined eighteen OCP residues in the breast milk of mothers from the Western Region of Bahia State, Brazil [40].

Hu et al. also revised the literature for POPs in breast milk in China for the last 20 years [41]. Olisah et al. reviewed the occurrence of OCPs in biological and environmental matrices in Africa for two decades [42]. In their review, data concerning the breast milk and OCP residues, particularly DDTs in breast milk samples, occurred at levels above the WHO stipulated limits, thus indicating a call for concern. Helou et al. also revised the OCP occurrence in human milk in Lebanon [15]. Kao et al. studied the possible association of OCP residues' occurrence in breast milk with negative effects and/or alterations in the

neurodevelopment of infants in Taiwanese mother-breastfed infant pairs, recommending further studies for the suggestive association [43].

Multiclass residue determination in both human and animal milk samples from the region of Iran has been recently reported [44]. Moreover, the OCP residues in bovine milk [18] and their possible human health risks have also been studied [45]. Levels of Polychlorinated Bisphenyls (PCB) and OCPs in donkey milk and the related toxicological risk to the consumer have also been studied recently [46]. Aydin et al. also analyzed milk samples for OCPs—among other pollutants—and determined the health risk of human exposure to organohalogenated pollutants (OHPs) [47], whereas OCP residues in animal milk (cow, buffalo, and sheep) in a region of Turkey have been studied [48]. The OCP residues in breast milk from a rural Armenian population in 1993–2000 have also been reported [49].

Kewsani et al. have also reviewed the global occurrence of OCPs, reporting also possible residues in milk [50].

3. Methods for Sample Preparation

Milk, due to its nature, is a complex matrix—high fat and protein content—for the analysis of pesticide residues. Due to the low concentration range of the residues and the low levels of detection required, efficient sample preparation methods are necessary. Martins et al. [51] reviewed the methods used for OCP determination in milk, reporting a time period of two decades. Therein, conventional extraction techniques along with other alternatives are reported: liquid–liquid extraction (LLE), solid-phase extraction (SPE), matrix solid-phase dispersion (MSPD), QuEChERS method (for Quick, Easy, Cheap, Effective, Rugged, and Safe), pressurized liquid extraction (PLE), and solid-phase microextraction (SPME), along with clean-up procedures to eliminate possible interference from co-extracted eluents. Among these procedures are freezing centrifugation, liquid–liquid partitioning, gel permeation chromatography, and SPE.

Herein, we report novel methods for OCP determination for the last decade. Among them, new materials (e.g., for SPME [52]).

Method Selection Criteria for Sample Preparation

The nature of the initial milk sample, solid or liquid, will define the sample preparation, as will also be pointed out in our review. Briefly, for solid samples, it is often necessary to pretreat them by sieving, grinding, or drying in order to avoid possible agglomeration of granules and for better dissolution with the solvent or the sorbent. Residual and/or water content should also be taken into consideration in the extraction processes.

In the case of liquid samples, the initial step is the filtration or centrifugation followed by the mixing with a selected solvent [53]. However, special treatment is needed in the case of special matrix composition. For example, for samples with high protein content, it may be necessary to first precipitate the proteins. For samples with high fat content, the fat is co-extracted with the pesticides. Thus, for instance, in vegetables with relatively high fat content, if lipids are not removed, they can be injected into the chromatographic system, resulting in lower sensitivity, shorter column life, and possible damage to the detection system [54].

Speaking of liquid matrices, traditional sample isolation procedures are used up to date; however, they are time-consuming, complex, expensive, and generate significant amounts of waste. For instance, for high-fatty matrices (>20% lipids), non-polar solvents are used to dissolve the fat and to extract the lipophilic pesticide residues, whereas in low-fatty food matrices having a low fat composition of 2–20%, such as milk, a wide polarity range is needed [55], depending on the target analyte. Recently, several new analytical techniques have been developed for sample preparation, extraction, and determination of pesticide residues that are more automated and environmentally friendly, thus reducing waste and large quantities of organic solvents in accordance with the principles of green chemistry. In the following sections, the main extraction methods used will be described.

4. Extraction Methods

4.1. Liquid–Liquid Extraction (LLE)

The liquid–liquid extraction (LLE) method is still one of the preferred methods for OCP extraction despite the age of this method and its drawbacks [56]. In brief, LLE methodology consists of shaking the samples of liquid milk several times together with the selection of specific organic solvents for the extraction of pesticide residues from the main matrix volume. In the event of pesticide isolation systems containing water, the water must be removed or separated. The solvents commonly used in this method are organic [57]. LLE is also offering excellent recovery and analytical precision [58]. The most commonly used solvents for pesticide isolation are ethanol, methanol, ethyl acetate, hexane, and their mixtures such as ethanol/ethyl acetate, acetone/hexane, ethyl acetate/acetone/methanol, hexane/dichloromethane, and petroleum ether [59]. The liquid–liquid extraction can be carried out in four different ways, namely discontinuous extraction, continuous extraction, countercurrent extraction, and direct extraction. Of the above, the most common of all is the discontinuous extraction, which is achieved in several stages [60]. Despite its broad usage, because of its simplicity, LLE has several drawbacks, such as prolonged experimental time, being labor-intensive, and not being environmentally friendly since large amounts of organic solvents are required [56].

Extraction of lipophilic compounds with LLE: The method has been applied recently in a study carried out in Nigeria where the concentrations of various OCPs were analyzed in six different evaporated milks. More specifically, the LLE method was used to determine the content of the samples of OCPs. Qualitative and quantitative measurement of OCPs was then performed using gas chromatography mass spectrometry (GC-MS). The reliability of the analytical procedures was tested in terms of percentance recovery (%R); the %R values for Heptachlor, Endrin, Endosulfan, and 4,4'-DDT ranged from 90% to 96% within the acceptable range of 70–110%, according to the EPA. The results of the measurements showed that phytopharmaceuticals -HCH, β -HCH, γ -HCH, d-HCH, Heptachlor, Heptachlor epoxide, Aldrin, Dieldrin, Endosulfan I, Endosulfan II, Endosulfan sulfate, p,p'-DDD, p,p'-DDE, p,p'-DDT, Endrin, Endrin aldehyde were found, and Endrin ketone and Methoxychlor at levels ranging from total OCPs 21,632 $\mu\text{g}/\text{mL}$ to 39,010 $\mu\text{g}/\text{mL}$ [61].

In the study by Klinčić et al., twenty (20) polychlorinated biphenyls (PCBs) and seven (7) OCPs have been analyzed in milk samples. Milk fat extraction is followed for the analysis of OCPs using Bligh and Dyer extraction (extraction of milk with a mixture of chloroform and methanol (1:1)) to extract about 5 g of unfrozen homogenized milk twice. Chloroform extracts were dried and separated under a nitrogen flow. Then it was cleaned up with sulfuric acid. Under nitrogen flow, cleaned extracts were dried. High-resolution gas chromatography with electron capture detectors was then performed using two capillary columns. Quantitative and qualitative analyses have been done by comparison with the external standard, and both columns have been used for the analysis of each sample. The reproducibility and recovery were determined by adding known amounts of all analyzed compounds to the aliquots of homogenized samples before the extraction. Average recoveries for the organochlorine pesticides in the range between 55% and 110% Method reproducibility expressed as % RSD was between 2% and 17% and 2% and 18% for organochlorine pesticides and PCBs, respectively [62].

4.2. Dispersive Liquid–Liquid Microextraction (DLLME)

In DLLME, the selected extraction solvent is mixed with a dispersive solvent, followed by the injection of the mixture into the aqueous sample [56]. Analytes are then instantly partitioned into the extraction phase, which is the emulsion that has been formed after the injection of the mixture. The large surface area due to the formation of microdroplets benefits DLLME compared to the classical LLE. On the contrary, several requirements need to be fulfilled for a successive application of DLLME, such as careful selection of the extraction solvent and optimization of several parameters, among them volume, ionic strength, pH value, extraction, and centrifugation time. There are a series of alternative modes in

DLLME, such as low-density solvent-based DLLME (LDS-DLLME). Air-assisted dispersive liquid–liquid microextraction (AA-DLLME), surfactant-assisted DLLME (SA-DLLME), uses surfactants as dispersive solvents [63], whereas cloud-point DLLME (CP-DLLME).

4.3. Solid-Phase Extraction (SPE)

The solid-phase extraction (SPE) method is a widely accepted method as an alternative extraction/purification method to the LLE method and is used to determine pesticides from liquid matrices. The process of the method is that initially the sample passes through a cartridge or a packed column that is filled with a solid adsorbent material, on the surface of which the analytes are adsorbed, while the other components of the sample pass through a special passage. The substances to be analyzed are adsorbed on the adsorbent material of the SPE and then eluted with an organic solvent [64]. Solid-phase extraction has several advantages that result in better results [65]. First of all, analytical procedure is much simpler as small volumes of solvents are used and much more pure extracts and higher recoveries are usually obtained. Also, this method allows avoiding the formation of an emulsion that is often found in liquid–liquid extraction and allows automatization. The adsorbents used in solid-phase extraction belong to three main categories: non-polar, polar, and ion-exchange substances. The choice of these materials depends on the food matrix.

In the article by Zheng et al., a multiresidue analysis of thirty (30) organochlorine pesticides in milk and milk powder was developed and applied for the analysis of commercial milk products. The samples were first extracted with a mixture of hexane and acetone and purified by gel permeation chromatography and solid-phase extraction (SPE). For the quantitative determination of organochlorine pesticides, data acquisition was performed by GC-MS/MS; a triple quadrupole mass spectrometer was used in selected reaction monitoring (SRM) mode. The quantification limits of all OCPs using the signal-to-noise ratio criterion (10/1) were calculated at 0.8 µg/kg. The quantification limits of all organochlorine pesticides except endrine are much lower than the maximum residue limits set by the European Union and China. Finally, the average recoveries ranged from 70.1 to 114.7%, with RSD below 12.9%. The method was applied to fifty milk and milk powder samples from a local market, and no OCPs were found [66].

4.4. Extraction Method Solid-Phase Microextraction (SPME)

The solid-phase microextraction (SPME) is an automated, easy, one-step, rapid solvent-free extraction method. This device consists of a fused silica fiber coated with a suitable stationary phase, where the analytes are adsorbed. Factors affecting the extraction steps include fiber type, extraction time, ionic strength, sample pH, extraction temperature, and sample agitation. The variables affecting the desorption steps include temperature, desorption time, and focusing oven temperature [64]. Depending on the position of the extraction fiber, three modes are used: direct extraction (d-SPME), headspace extraction ((HS)-SPME), and membrane protection extraction. SPME is a non-exhaustive extraction technique; thus, calibration for quantitative analysis is very important [67]. The method merits the advantages of simplicity and effectiveness in rapid sampling [68] and fiber reusability [67]. The SPME method also produces relatively pure and concentrated extracts and is ideal for mass spectrometry (MS) applications. During the application of the method, the clogging phenomenon that occurs in the SPE method does not occur. Moreover, SPME provides the advantages of short sample preparation time, small sample volume requirements, sampling over a range of matrices—from gaseous to liquid and solid—and solvent consumption elimination [69] and generally has simple, fast, and solvent-less features [70]. Moreover, HS-SPME has been successfully applied to a several range of matrices, from liquid samples and honey to olive oil for the determination of organophosphorus insecticides [71,72].

Experimental design through the statistical approach of a Response Surface Methodology (RSM) [73] and the design strategies to estimate and evaluate surface response—e.g., Box–Behnken design (BBD) [74]—is expected to indicate several crucial factors for this multiparameter-dependent procedure. For instance, Doehlert design has been applied in

the optimization of the extraction conditions in the determination of OCPs in bovine milk samples by headspace solid-phase microextraction (HS-SPME) [75].

Efforts are taken in order to develop and validate new materials for SPME. Taçaç et al. [76] utilized montmorillonite nanocomposite incorporated with natural biopolymers and benzyl functionalized dicationic imidazolium-based ionic liquid-coated fiber to develop an SPME coating for OCPs. The authors compared the extraction and analytical performance of the newly developed HS-SPME method coupled with GC/MS or GC/ECD to published works for OCP determination in different sample matrices, and the applicability in different sample matrices—milk among them—was studied.

Fernander-Alvarez et al. [77] analyzed bovine and powdered milk, where more than thirty organochlorine pesticides and pyrethroids were found and analyzed. More specifically, a solid-phase microextraction (SPME) technique followed by gas chromatography with microelectron capture detection (GC-ECD) was developed. Dispersive solid-phase microextraction (DSPME) was performed first using a (PDMS)/(DVB) coating. This method had high sensitivity and fine linearity, with limits of detection (LOD) at the sub-ng mL^{−1} level. R.S.D. were also evaluated <15% within a day. It has also been obtained high recoveries (>80% in most cases) for different types of milk. Powdered milk (PM) certified reference material was used. These were quantified using external calibration and standard addition protocols.

4.5. Gas-Diffusion MicroExtraction (GDME)

GDME is a technique developed almost a decade ago [78,79]. Through gas diffusion (GD), which is a subclass of the membrane-based techniques for analyte extraction, analytes are separated from sample solution through a gas-permeable membrane, and the microextraction (ME) is achieved since the volume of the extracting phase is very small compared to the volume of the sample. The method was first applied, combined with derivatization, for the determination of vicinal diketones [79] and acetaldehyde (AA), methylpropanal (MA), and furfural (FA) [78] in beer.

The GDME method was applied once, by Lobato et al. [80], for the direct extraction of OCPs (lindane, α - and β -hexachlorocyclohexane, hexachlorobenzene, p,p'-DDE, aldrin, dieldrin, and α -endosulfan) from ultra-high temperature processing (UHT) semi-skimmed milk samples.

Analytes were afterwards detected by gas chromatography with electron capture detection (GC-ECD) and mass spectrometry (GC-MS) with an ion trap mass detector; additionally, GC-MS analysis was performed in two different modes: SIM and MS/MS mode. The analytical characteristics of the method were evaluated using blank milk samples with known amounts of OCP (spiked samples). The method was linear—the coefficients of determination (r^2) ranged from 0.991 to 0.995—for all the above OCPs. The reported limit-of-detection (LODs) ranged from 3.7 to 4.8 $\mu\text{g L}^{-1}$. The method also had very good precision—the RSD values were lower than 10%—and high accuracy—the reported recoveries ranged from 71% to 99%. The authors analyzed six milk samples using GDME-GC-MS in SIM and MS/MS mode. For confirmation of possible OCP occurrence, one OCP (aldrin) was detected in one sample, below the LOD—lower than the MRL set in EU legislation. For aldrin and dieldrin (aldrin and dieldrin combined expressed dieldrin), MRL in milk is 0.006 mg kg^{−1} [81].

As reported by the authors, “although the GDME system is not commercially available it can be easily ‘home-made’ produced”, having the benefits of a low-cost sampling method that is also a greener alternative to other sampling systems.

4.6. Stir-Bar-Sorptive Extraction (SBSE)

The stir-bar-sorptive extraction (SBSE) method is a relatively developed technique based on the absorptive interaction of the compounds of interest with a polydimethylsiloxane coating deposited on a magnetic glass. The mechanism of operation of this method and the advantages are similar to those of the SPME method, but the enrichment factor,

which is determined by the amount of extracted phase, is up to 100 times higher. Therefore, the SPME method is considered ideal for the detection of compounds that present high concentrations, while the SBSE extraction method is ideal for trace analysis. As in the SPME method, the analysis rod can also be used to sample the volatile and non-volatile substances above the sample. This method can also be used for liquid or semi-solid composite matrices and therefore has potential for many applications in food analysis. This technique is ideal for isolating less polar compounds. Its applications are increasing in food analysis, but due to the aforesaid limitations, its applications are currently limited to non-fatty food matrices and non-polar or semi-polar analytes [82].

Li et al. [83] reported the analysis of eight organochlorine pesticide residues in environmental and biological matrices—among the matrices, raw milk was included. More specifically, the method used was hollow fiber-stir bar sorptive extraction (HF-SBSE) coupled with gas chromatography-mass spectrometry with ion ionization mode for the determination. Standards and samples were analyzed in selective ion monitor (SIM) mode. For the HF-SBSE, the ZrO_2 nanoparticles were deposited on the surface of SiO_2 microspheres, resulting in composite microspheres with expected enhanced extraction efficiency. The results showed good linearity ($r > 0.999$) for all the calibration curves. The recoveries were in the range of 69.0 to 114.1%, and the RSD% values were less than 10.2%. In raw milk, the LOQs ranged from 0.0010 to 0.0090 $\mu\text{g ml}^{-1}$ and the LODs from 0.0003 to 0.0030 $\mu\text{g ml}^{-1}$. Finally, in the raw milk samples, the % recovery ranged from 73.2 to 104.7. Analysis of raw milk samples using the proposed method showed—in liquid samples no pretreatment was used—values of p,p' -DDE, p,p' -DDD, and o,p' -DDE below LOQ, 0.100 and 0.100 mg kg^{-1} , respectively.

4.7. QuEChERS Method (QUick Easy ChEap Robust Safe)

The QuEChERS method is a fast and convenient replacement for the liquid–liquid extraction method that provides high-quality results in a minimal number of steps and with low consumption of solvent and glassware. The name of this method means fast, easy, cheap, effective, durable, and safe [84]. This method allows the simultaneous extraction of polar and non-polar compounds with sufficient recoveries and thus becomes suitable for the isolation of a wide range of compounds. It is mainly used in non-fat samples. The advantages of this method are high recovery rates, accurate results, high sample throughput, low solvent and glassware usage, less labor, lower reagent costs, and durability. The main disadvantage of the method is the lower concentration of the target compounds in the final extract than the one in concentrated extracts obtained using most traditional procedures. Consequently, to achieve the necessary sensitivity and the desired limits of quantification, the final extract must be concentrated to a greater extent [64]. Difficulties may also arise when the official methods are applied to emulsified fatty foods such as milk. Some lipophilic pesticides, such as DDE and hexachlorobenzene HCB, have been proved to be poorly recovered from milk samples, and the percent recovery tended to decrease as the fat content increased [85].

Mekonen et al. [34] analyzed human breast milk from 168 mothers from southwestern Ethiopia. These milk samples were collected at three time points, which were baseline (1st month), midline (6th month), and end line (12th month). OCPs were extracted using the QuEChERS method, followed by the dispersed solid-phase extraction cleanup method. For the quantification, identification, and separation, the GC-ECD method was used. Eight organochlorine pesticides were analyzed in 447 breast milk samples. The LOD expressed per gram milk fat bases were 0.018–0.078 $\mu\text{g/g}$ milk fat and the LOQ were 0.062 to 2.38 $\mu\text{g/g}$ milk fat. The coefficient of determination (r^2) calculated was > 0.99 for all the pesticides in the study.

DDT was the pesticide that had the biggest concentration with 2.25, 1.68, and 1.32 $\mu\text{g/g}$ milk fat at three time points (baseline, midline, and endline), respectively, which was above the daily intake set by FAO/WHO. p,p' -DDE and p,p' -DDT at a mean concentration of 1.85 mg g^{-1} milk fat (taking into account 3.5 g of fat per 100 mL of breast milk) (above

WHO/FAO MRL of 0.02 mg g⁻¹ of milk fat) in all milk samples; p,p-DDD at 241 samples from 0.08 to o,p-DDT at 191 samples was reported. The DDT/DDE ratio was < 5 (indicating historical exposure to DDT).

A multi-class method of 50 pesticide residues (15 of them OCPs) in 60 milk samples—raw (15 samples), pasteurized (36 milk samples with lipid content varying from 1 to 3.3 fat percent), and powdered (eight of them powdered) cow samples and human milk from 15 volunteer women from Iran, using ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) using a triple quadrupole mass analyzer in electron spray ionization mode, and for the OCPs using gas chromatography—electron capture detector (GC-ECD)—the CG/MS with ion trap analyzer in electron ionization mode was also used for the confirmation of CG/ECD results—has also been reported recently [44]. Analytes were extracted using modified QuEChERS extraction based on the AOAC official method [55] with some modifications [86] followed by SPE cleanup. The method was validated for the OCPs using GC/EDC analysis through linear dynamic range, LOD and LOQ, and % matrix effect. The linear dynamic range was reported from 0.0005 to 0.5 mg kg⁻¹ up to 0.003 to 0.5 mg Kg⁻¹ for β-HCH and p,p'-DDT. The % matrix effect ranged from -9 for δ-HCH to -47 for p,p'-DDT. According to the authors results, no pesticide residue was determined in all thirty-six pasteurized milk samples and eight powdered milk samples. Among humans, contaminated samples contained 0.011 mg kg⁻¹ p,p'-DDT, 0.025 mg kg⁻¹ p,p'-DDE, and 0.0016 mg kg⁻¹ p,p'-DDD. Only p,p'-DDE residue levels in three human milk samples exceeded the EU MRLs. The authors also used Monte-Carlo simulation for the assessment of the health risk, concluding that adults are not at significant non-carcinogenic and carcinogenic risk. As also reported by the authors, in children, due to persistence, organochlorine pesticide residues in a few human milk samples can create the health of children with a severe audience.

5. Solid or Semi-Solid Matrix

5.1. Soxhlet Extraction (SE)

When the matrix is solid or semi-solid, there are different techniques used to isolate the pesticides, as they have better yields and better isolation for the substances that we need to determine.

The SE is a widely used method suitable for the extraction of semi-volatile organic compounds from solid samples. A commercially available automatic SE apparatus is available as Soxtec extraction [87]. SE is used for fat extraction from different matrices. [88]. As for milk, it is used for milk powder samples. Usually n-hexane is used, but other solvent systems can also be used [51]. An advantage of the SE method is that after the leaching step no filtration is required, and due to the low cost of basic equipment, sample throughput can be increased by simultaneous parallel extraction [88]. There are several modes developed: conventional SE, high-pressure SE, automated SE, ultrasound-assisted SE, and microwave-assisted SE [89]. Conventional SE methods include large amounts of solvent and prolonged extraction time, followed by the evaporation and clean-up before the analysis, which are the major drawbacks of the method. Moreover, the prolonged extraction time along with the extraction of target species at the solvent boiling point may result in thermal decomposition of thermolabile compounds [88]. High-Pressure SE filled with CO₂ as extractant had been used for the non-polar organic compounds OCPs and polychlorinated biphenyls (PCBs) in various matrices (certified potato, carrot, olive oil, and lyophilized fish tissue), obtaining high extraction efficiencies after 30 min for the lipid matrix of, e.g., 0.3 g of butterfat reference material [90]. Conventional SE using a mixture of hexane/acetone in a volume ratio of 2:1 had been used for the residue determination of 13 OCPs in high-fatty products (butter and margarin) [91] and a Soxtec method using petroleum ether for 16 OCPs in fish feed samples [92].

Brunetto et al. studied the levels of DDT residues in 145 breast milk samples of Venezuelan women. Samples were lyophilized, and SE was applied using n-hexane. After cleanup, due to saponification of the extract, using a Florisil microcolumn, high-

performance liquid chromatography with diode-array detection (HPLC-DAD) was applied for the quantification [93]. For spiked samples, the recovery rate ranged between 99% and 107%. Finally, the LOD of the method was $0.5 \mu\text{g L}^{-1}$. For the total of the analyzed whole milk samples, all participants had a residual level of DDT from 5.1 to $68.2 \mu\text{g/L}$, reflecting its extensive use and the widespread low-level contamination. Also, Prado et al. analyzed 37 and 26 HM samples in the urban and suburban zones, respectively, of Mexico City between 1997 and 1999 using SE followed by GC-ECD [94].

Zhou et al. [95] used SE for the analysis of OCPs in HM of mothers in China. In their national survey, 1237 breast milk samples from 12 provinces in China were collected combined to 24 pooled samples of HM. HM samples were freeze-dried, SE extracted with a mixture of n-hexane and dichloromethane (1:1, *v/v*) (24 h), and cleaned up by gel permeation chromatography (GPC). Detection was performed by gas chromatography-negative chemical ionization-mass spectrometry (GC-NCI-MS) with a VF5-5MS capillary column and helium as carrier gas. Twenty-three OCP compounds were quantified using the isotope dilution method. Recoveries of the analytes for two spiked levels (2.0 and 10 ng g^{-1}) ranged from 85% to 130% and relative standard deviations (RSDs) were $< 10\%$. Limits of detections (LODs) for *a*-, *b*-, *g*-, and *d*-HCH were 1.2, 1.8, 2.3, and 3.0 ng g^{-1} lipid, respectively. The LODs for *pp'*-DDT, *op'*-DDT, *pp'*-DDE, *op'*-DDE, *pp'*-DDD, and *op'*-DDD were 3.0, 1.2, 0.5, 0.8, 1.2, and 1.0 ng g^{-1} lipid, respectively. The LOD for HCB was 0.05 ng g^{-1} lipid. The LODs for other OCPs were 0.5 to 3.0 ng g^{-1} lipid. A wide range of concentrations for the 23 OCPs compounds were detected in the 24 pooled samples of breast milk from $<\text{LOD}$ to 1.660 ng g^{-1} lipid. DDTs, HCHs, and HCB were detectable in every pooled sample, and their concentration varied from 153.6 ng g^{-1} lipid to 1756.3 ng g^{-1} lipid (mean, 527.2 ng g^{-1} lipid); 55.8 ng g^{-1} lipid to 536.4 ng g^{-1} lipid (mean, 231.8 ng g^{-1} lipid); and 18.4 ng g^{-1} lipid to 56.8 ng/g lipid (mean, 32.8 ng/g lipid), respectively.

5.2. Pressurized Liquid Extraction (PLE)

The modern extraction technique pressurized liquid extraction (PLE), also known as accelerated solvent extraction (ASE) or pressurized fluid extraction (PFE) [96], or pressurized hot solvent extraction (PHSE) or high-pressure solvent extraction (HPSE) or high-pressure high-temperature solvent extraction (HPHTSE) or subcritical solvent extraction (SSE) [97,98]. Shortly after its introduction in the middle of 1990, it was approved as an Environmental Protection Agency (EPA) method for the determination of semi-volatile organic compounds, among them organochlorinated pesticides, in solid and semi-solid environmental samples [98].

As also reported previously [51,99], PLE is a fast extraction technique, overcoming the disadvantages of other extraction methods, such as the long processing time of the samples and the consumption of large volumes of organic solvents [100]. Thus, its main advantages compared to “classical” solvent extraction are smaller extraction times and solvent use, a higher yield of contaminants, and improved selectivity; however, for optimum and high-quality results, the operating conditions should be optimized for each matrix [101]. PLE has high extraction efficiency for OCPs, slightly higher for some more volatile compounds (e.g., HCB) as compared to the classic Soxhlet procedure at a high lipid matrix such as fish [102].

The PLE utilizes high pressure in order to reach subcritical temperature conditions for the extraction of the contaminants [96]. The combination of high temperature and pressure results in better extraction efficiency, thus minimizing the use of solvents and helping to speed up the extraction process [99]. Several parameters are usually optimized for the extraction efficiency, such as the extraction temperature, the number of static cycles, and the extraction solvent mixing. Moreover, in the milk matrix, two objectives should also be studied and optimized: the recoveries and the minimization of the fat content in the final extract [100].

As also reported above, a Response Surface Methodology (RSM) and the design strategies to estimate and evaluate surface response—e.g., Box–Behnken design (BBD) [74]—are expected to indicate several crucial factors for this multiparameter-dependent procedure.

According also to cited literature [96], the integration of machine learning and language models has the potential to provide insights for the selection of solvents and absorbents.

The PLE has been successfully used for the determination of pesticides in different food matrices, with particular interest in applying this technique in food analysis for food matrices that contain lipids, among them milk samples. The advantages of this method, as mentioned above, are the small amount of solvent consumed and the short time needed for the extraction [64]. PLE is commonly used for solid and semi-solid samples. It also employs solvent extraction at elevated temperatures and pressures, always below the respective critical points, so as to maintain the solvent in a liquid state throughout the extraction procedure [98]. On the contrary, as reported from Chung et al. at their critical review [99], the initial cost of the method is increased as large quantities of undesirable substances contained in the sample matrix are coalesced with certain unstable organochlorinated pesticides such as aldrin, dieldrin, endrin, and pentachlorobenzene, yielding low recoveries. Another disadvantage of PLE is the necessity of cleaning the samples after extraction, and particular attention should also be paid for possible degradation or unstable compounds.

On the other hand, compared to various other extraction techniques such as microwave-assisted extraction (MAE), magnetic solid-phase extraction (MSPE), matrix solid-phase dispersion (MSPD), QuEChERS, ultrasonic-assisted extraction (UAE), supercritical fluid extraction (SFE), dispersive solid-phase extraction (dSPE), SPME, SBSE, stir-bar sorptive-dispersive microextraction (SBSDE), and dispersive liquid–liquid microextraction (DLLME), PLE yields better extraction percentages with appropriate selectivity for removing matrix constituents [96].

As reported also by Soriano et al. [96], nowadays PLE is applied in complex food matrices such as those having high lipid and/or protein content that strongly retain analytes.

The first development of the PLE technique, with on-line cleanup followed by GC-MS/MS, for the multiresidue analysis of twelve organophosphorus and organochlorine pesticides in infant formulas has been reported by Mezcua et al. [100]. The analysis took place using a GC-MS/MS system using electron ionization and equipped with a CP-8400 autosampler. For the MS analysis, typical MS/MS parameters were optimized for each compound—for the fragmentation of the precursor ions, collision-induced dissociation (CID) in the non-resonant (intermolecular) excitation mode was performed for all the pesticides. The method was validated having very good analytical characteristics—mean recoveries for spiked samples ranged from 70 to 110% for the OCPs having good RSD values (70% to 110%). The method was applied to a pilot monitoring study of twenty-five samples of powdered infant formulas in Spain. The authors reported five positive findings for the two cyclo-diene stereoisomers of endosulfan, Endosulfan I and II, at a concentration range of 1.60 to 5.03 $\mu\text{g kg}^{-1}$ and of 1.18 to 2.70 $\mu\text{g kg}^{-1}$, respectively. No positive finding was reported for endosulfan sulfate.

5.3. Matrix Solid-Phase Dispersion (MSPD)

The matrix solid-phase dispersion (MSPD), first introduced by Barker et al. back in 1989 [103], is an extraction and purification technique bearing the advantages of SPE, but it is applied for the isolation of residues through blending of solid chemical support in solid matrices—whereas SPE is applied to liquid matrices—eliminating the need for sample homogenization and matrix debris removal [104] before column application [103].

The method is based on the blending of the matrix with an inert sorbent; in its first introduction [103], the matrix was a biological sample—a tissue- and a bonded-phase solid support material that induces disruption and, as a “bound” solvent, dispersion of the sample over the surface of the bonded-phase support material [105].

The method is applied through the grounding of the homogenized samples with the solid sorbent—usually used dispersants are reversed-phase sorbents such as octadecyl-silica (C_{18}), but also normal-phase non-bonded sorbents such as Florisil, alumina, and silica have been proposed, as well as mixtures such as silica gel and charcoal—in order to finally achieve a homogenous distribution of the raw material with the sorbent particles [51].

Recently, novel sorbent materials have been introduced, among them Molecularly Imprinted Polymers (MIPs)—e.g., surface MIPs for penicilloic acid and penilloic acid in milk [106], graphene-based sorbents, and metal-organic framework materials [107].

Generally, in a typical MSPD procedure, samples are blended with the sorbent, transferred, and packed into an extraction column [107]. After column packing, elution is applied. In Figure 2, a schematic representation of the main experimental parameters to be mainly considered in method development is illustrated. The nature of the eluent is critical to achieving effective desorption of pesticides from the adsorbent material [51].

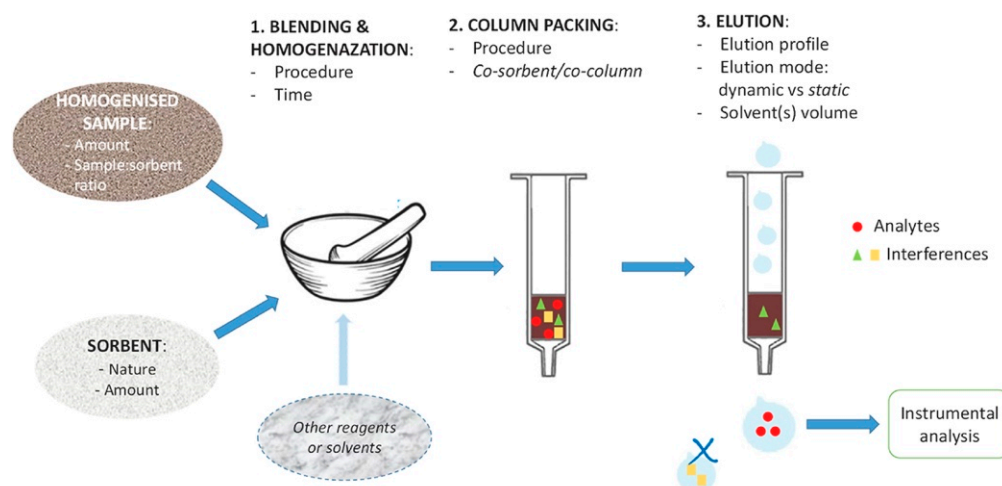


Figure 2. Schematic representation of the three main steps in the MSPD method development (experimental parameters to be optimized are indicated, whereas the optional ones are displayed in italics) (from [108], adapted with permission from the publisher).

The simplicity and straightforward nature of the MSPD process resulted in its rapid acceptance for its application in a series of matrices, from solid and semi-solid to viscous and liquid samples. The evolution of novel materials and their possible incorporation as dispersant sorbents is facilitating new method developments and application studies. [108]. The several innovations that were incorporated resulted in the merits of the method: simplicity, flexibility, rapidity, robustness, and straightforward nature [109].

Furusawa [110] used five polar sorbents for the normal-phase MSPD of aldrin, dieldrin, and DDTs in animal fats, and analysis was performed with an HPLC -(C₁-silica) column and a mobile phase of 50% (*v/v*) ethanol (in water) solution—using a photodiode array (PDA) detector.

MSPD using octadecyl-(C18)-bonded silica had been applied for the multiresidue determination of OCPs and polychlorinated biphenyls in milk samples by CG-ECD [104]. Their method was a modification—sample volume, polarity of elution solvent, alumina for clean-up, and extension to six PCBs—of a previously reported method [111] and was validated by determining the accuracy (% recovery), the precision (repeatability and reproducibility), and sensitivity (limits of detection and quantification). In spiked milk samples for the spiking levels of 10 µg L⁻¹ and 1 µg L⁻¹, the recovery values and %RSD were in the range of acceptable limits from 79% to 99% and 74% to 109%, respectively, with the exception of β-Endosulfan and Endosulfan-sulfate that were not recovered. Detection limits for OCPs in milk ranged from 0.02 to 0.62 µg L⁻¹, and twenty-five milk samples (UHT liquid whole milk) were analyzed.

Lehotay et al. [55] evaluated and applied two rapid methods for sample preparation and analysis of fatty foods, including milk. The first method used was QuEChERS, and the second was MSPD (matrix solid-phase dispersion). Both method extracts were analyzed by gas chromatography/mass spectrometry and liquid chromatography/tandem mass spectrometry. Electron ionization was applied in the CG/MS apparatus running mainly in Selected Ion Monitoring (SIM) mode, and the liquid chromatography tandem mass

spectrometry (LC/MS/MS) consisted of a triple quadrupole instrument using electrospray ionization. For two concentration levels of low and high spiking levels, 50 and 500 ng g⁻¹, respectively, and the recovery and %RSD values for chlordane, DDE, dieldrin, endosulfan sulfate, heptachlor epoxide, hexachlorobenzene, and lindane in milk were found in the commonly acceptable limits ranged from 70% to 120% and %RSD below 15%, with the exception of DDE, dieldrin, endosulfan sulfate, heptachlor epoxide having a recovery value and %RSD of 121(6), 133(15), 155(5), 127(9), respectively, for the concentration level of 50 ng g⁻¹ and hexachlorobenzene having a recovery value and %RSD of 65(8) for the concentration level of 500 ng g⁻¹. As concluded by the authors, the MSPD method had somewhat higher recoveries of the most lipophilic pesticides fortified in the case of milk.

Analysis of incurred reference materials or proficiency testing should be performed in order to obtain a more trustworthy conclusion, especially for lipophilic pesticides such as OCPs. Moreover, for low-fatty foods (2–20% of fat content), such as milk that contains ~3% fat content, QuEChERS also performs well for the extraction of lipophilic pesticides [55].

5.4. Solid–Liquid Extraction (SLE)

The SLE is based on the ratio of the analyzer distribution between solid and fluid. The samples commonly used in this method are solid, and especially for milk powdered milk is used. The same process has been applied to liquid milk, which was first mixed with anhydrous sodium sulfate and turned into powder. So, in the case of liquid/liquid extraction, the selection of the appropriate solvent is a critical factor to get the best results during this method. The main solvents used in this method are dichloromethane and light petroleum.

Frías et al. determined organochlorine compounds in four types of biological samples, among them human milk as well as infant formula milk using LLE or SLE, followed by HPLC-DAD clean-up and analysis with GC-MS/MS. The MS apparatus consisted of an ion trap spectrometer that was operated in the electron ionization mode, and the option MS/MS was used. The method was validated, and the % recovery and %RSD intraday values were in the acceptable range, having values from 76(6) to 110(10) for endosulfan lactone and 4,4' DDE, respectively.

In their proposed methodology, eighteen (18) OCPs were studied, along with PCBs, in five (5) milk samples—among other samples. In one formula milk, the authors documented 4,4' DDT below its LOQ. In maternal milk samples, 4,4' DDE was detected at concentration levels from its LOD to 0.007 mg L⁻¹, from its LOD to 0.020 mg L⁻¹, and from its LOD to 0.0028 mg L⁻¹ in transition milk, colostrum milk, and mature milk, respectively. Mirex, 4,4' DDT, and lindane were also detected in maternal milk [112].

5.5. Microwave-Assisted Extraction (MAE)

Firstly introduced as microwave extraction by Ganzler et al. [113] in their pioneering work where microwave irradiation was used for the extraction of several types of compounds from different matrices, MAE has gained more attention recently in sample preparation techniques [114].

In food and environmental analysis MAE is used in trace pesticides, which is one of the three main categories for analysis—the other two being aromatic fused-ring compounds and trace pharmaceuticals and hormones [115].

For complex matrices, MAE can be an alternative extraction technique to conventional ones for solid–liquid or liquid–liquid extraction procedures and can also compete to recent techniques such as ultrasound-assisted extraction (UAE), PLE, and ASE, having the advantages of a significant reduction in time and solvent consumption [115]. Nevertheless, applied temperature should be taken into consideration for the extraction of thermolabile pesticides [114].

MAE are electromagnetic waves (frequency from 300 MHz up to 300 GHz), and the direct effect of this microwave radiation through the microwave energy is the heating of the sample through ionic conduction and dipole rotation. Thus, depending on their

dielectric coefficient materials, in fact increasing absorption of microwave energy upon increased dielectric constant (ϵ')—thus solvent and solid matrix of a sample—show a different behavior [116]. The extraction heating process occurs mainly by two mechanisms: the sample could be immersed in a solvent or mixture of solvents that absorb microwave energy strongly, or in a combined solvent with both high and low dielectric losses. The dielectric loss (ϵ'') is defined as a measure of the efficiency of the solvent to dissipate the absorbed microwave energy into heat, and the dissipation factor ($\ln \delta = \epsilon''/\epsilon'$) determines the efficiency of the extraction process [117]. Moreover, samples with high dielectric loss can be extracted with a microwave-transparent solvent (e.g., hexane).

The main advantages of MAE are the reduction of extraction time, the significant reduction of organic solvent consumption, and the possibility of running multiple samples. [118]. Parameters that affect MAE efficiency are solvent nature and volume, extraction temperature, extraction pressure and time, and irradiation power [117]. Matrix characteristics play a crucial role in the optimization of MAE. Thus, the water content of the sample, due to its high dielectric constant, heats up very efficiently and facilitates the contact of the sample with the extraction solvent, resulting in the release of the analyte(s) into the extraction solvent. On the contrary, excessive water could negatively affect the extraction through the possible formation of a protection layer and/or leading to solubility issues in the extraction of apolar analytes through apolar solvents [114].

Two technologies are mainly used: pressurized MAE—performed in extraction cells—and focused MAE—an open MAE considered more suitable for extracting thermolabile compounds, and several modifications of the above have been proposed and developed, such as on-line MAE, dynamic MAE (DMAE), and ultrasonic MAE (UMAE). Moreover, microwave-assisted micellar extraction (MAME), ionic liquid-based MAE, and microwave-assisted aqueous-solution extraction have been proposed [115].

Several solvents and mixtures have been tested for OCPs in lipid matrixes, such as n-hexane for OCPs in fatty tissues of marine mammals and spiked port fat [119], ethyl acetate-cyclohexane (1 + 1, v/v) in fatty fish tissue [120], and hexane-acetone (8:2, v/v) in fish [121].

Pare et al. [122] back in 1997 proposed the usage of MAE for the extraction of fat from dairy, among others, products. Although the method was applied for the determination of OCPs in mussel tissue [123], fish muscle [124–126], and fish tissue [120], for pesticides in infant milk, and for polybrominated diphenyl ethers in human milk [127], to the best of our knowledge, up to date it has not been applied for the extraction and determination of OCPs in human milk.

Fang et al. [128] used infant milk as a matrix for pesticides' determination. More specifically, an extraction method was developed consisting of both microwave-assisted extraction (MAE) and solid-phase extraction (SPE) to extract from infant milk formula pesticides of different polarities. MAE in a microwave vessel was used for the extraction of the analytes using 0.1% water in methanol at pH 12, and the extracts are preconcentrated with SPE. Extracts were analyzed with LC-MS/MS through electron spray ionization (ESI), and analyses were performed using a C-18 column for the LC system and the MS triple quadrupole mass spectrometer for multiple reaction monitoring (MRM) in positive ion mode. Experimental design—Derringer desirability function—was performed for the optimization of the extraction process. The relative standard deviations (RSDs) were found to be less than 8%. The extraction recoveries obtained ranged from 72% to 111% for all pesticides at a fortification level of 5–100 $\mu\text{g kg}^{-1}$.

6. Commenting on the Methods

In conclusion, although the possibilities of solid–liquid extraction present many advantages, there are certain characteristics that require improvement. The main problems to overcome are the substantial variation in performance of the products offered by different manufacturers, the small sample volume that can only be run with some SPE adsorbents, and finding an adsorbent and eluent that will analyze multiple residual compounds with a

very wide range of physicochemical characteristics. There is a plethora of methods for the extraction of OCPs. Table 1 presents the main pros and cons of using the afore-reported extraction methods in milk samples.

Table 1. Pros and cons using several extraction techniques in milk samples.

Extraction Method	Pros	Cons	Type of Milk ¹	Selected Citations for Application for OCPs in Milk
Liquid matrix				
LLE	- Simple method - Quick method	- Old and slow method - Labor intensive - Environmentally unfriendly	AM	[61]
SPE	- Simple analytical procedure - Small volumes of solvent used - High recovery method - Allows atomization - Avoidance of emulsion formation	- Not appropriate method for solid samples - Variation in performance of the product offered - Small sample runned exclusively by SPE absorbents - Difficulty finding absorbent and eluent with wide range of physicochemical characteristics	AM	[66]
SPME	- Good analytical performance characteristics - Simplicity of the method - Low-cost method - Pure and concentrated extracts produced - No organic solvents are used	Many factors affect the efficacy of this method	HM	[34]
			AM	[77]
GDME	Large surface area (compared to LLE)	- Careful selection of the extraction solvent - Need optimization of several parameters (volume, ionic strength, pH value, extraction, and centrifugation time)	HM	[80]
SBSE	- Ideal method for trace analysis - Wide range of matrices can be used - Successful isolation for less polar compounds	Limited application for non-fatty, non-polar, and semipolar analytes	RM	[83]
QuEChERS	- High quality result method - High recovery rates - Accurate results - High sample throughput - Low solvent and glassware usage - Less labor - Lower reagent costs - Durability	- Lower concentration of the target compounds - Poor recovery for high fat concentration milk	HM	[34]

Table 1. Cont.

Extraction Method	Pros	Cons	Type of Milk ¹	Selected Citations for Application for OCPs in Milk
Solid or semi-solid matrix				
SE	• Suitable method for solid food samples • In contact with fresh portions of extractant throughout the process • The temperature of the system remains high • No filtration is required • Sample throughput can be augmented by simultaneous parallel extractions • Inexpensive equipment	• Large amounts of solvent required • Time-consuming method • Increased possibility of thermal decomposition • Not an easily automated method	HM	[95]
PLE	• Small solvent consumption • Quick extraction	• Coalescence of undesirable substances causing low recoveries • Necessity for cleaning samples after extraction • High extraction temperature cause degradation of the compounds		
MSPD	• Rapid exchange of samples • Requires small sample and solvent size • Reduces environmental pollution • Improves work safety • Low cost per sample extraction • No expensive instruments required	• Careful selection of the dispersive sorbent and the elution solvent		
SLE	• Appropriate method for solid samples	• Not suitable method for liquid samples • False selection of solvent can cause false results		
MAE	• Low temperature requirement • High extraction efficiency • Full atomization • Ability to extract multiple samples without interference	• Lack of selectivity of substances needs to be isolated • Co-extraction of interfering compounds		

¹ HM: Human milk, AM: animal milk.

Application for the Determination of OCPs in AM and HM

Table 2 displays the techniques that were used for the development of methods in order to determine and/or monitor the OCP levels in milk samples.

Table 2. Methods and techniques for the determination/monitoring of possible OCP residues' occurrence in milk samples.

Technique(s) Used [†]	MS Apparatus and/or Ionization Mode	Sample Preparation ^{††}	Matrix	OCP Used for Method Development/Application	LOD/LOQ (mg kg ⁻¹)	Monitoring/Measurement in Milk Samples (Number of Samples/Number of OCPs above LOD)	Reference
GC-MS	(a)	LLE	HM	-HCH, β -HCH, γ -HCH, d-HCH, Heptachlor, Heptachlor epoxide, Aldrin, Dieldrin, Endosulfan I, Endosulfan II, Endosulfan sulfate, p,p'-DDD, p,p'-DDE, p,p'-DDT, Endrin, Endrin aldehyde		Six samples/ eighteen OCPs for method/ three major classes of OCPs detected	[61]
UHPLC-MS/MS and GC-ECD	triple quadrupole mass analyzer	QuEChERS and SPE	HM and AM	Fifteen OCPs for method development/ p,p'-DDT, and p,p'-DDD, p,p'-DDE	0.00015–0.0009 /0.0005–0.001	Thirty-five commercial and fifteen raw milk samples/p,p' DDE above MRL (FAO and WHO) in three human milk samples, other two OCPs below MRL	[44]
GC-ECD		SPE	M and PM	Sixteen OCPs and metabolites (α -chlordane, methoxychlor, γ -chlordane, endrin ketone, aldrin, α -lindane, β -lindane, γ -lindane, δ -lindane, 4,4'-DDD, 4,4'-DDE, 4,4'-DDT, dieldrin, endosulfan I, endosulfan II, endosulfan sulfate, endrin, heptachlor, heptachlor epoxide isomer B). α - and β -hexachlorocyclohexane, lindane, hexachlorobenzene, p,p-DDE, aldrin, dieldrin, and α -endosulfan	0.010–0.52 /0.003–0.16 ng mL ⁻¹	Thirty-five raw milk samples/chlordane at 1 ng mL ⁻¹ at one sample	[77]
GC-ECD and GC-MS	Ion Trap Mass Detector in SIM and MS/MS mode	GDME	HM	α - and β -hexachlorocyclohexane, lindane, hexachlorobenzene, p,p-DDE, aldrin, dieldrin, and α -endosulfan	LODs: from 3.7 to 4.8 μ g L ⁻¹	Six milk samples /Aldrin in one sample below the LOD	[80]
GC-MS	Electron Ionization/SIM mode	HF-SBSE	RM	α -BHC β -BHC γ -BHC δ -BHC p,p'-DDE p,p'-DDD o,p'-DDT p,p'-DDT	LOD: 0.0003 to 0.0030 (μ g mL ⁻¹)/LOQ: 0.0010 to 0.0090 (μ g mL ⁻¹)	(b) /p,p'-DDE detected, but could not be quantified, p,p'-DDD and p,p'-DDT at 0.100 mg kg ⁻¹ (each of them) 447 (at three sampling times)/only DDT and its metabolites were detected, total DDT concentrations at baseline (1st month), midline (6th month), and end line (12th months) were 2.25, 1.68 and 1.32 μ g g ⁻¹ milk fat, respectively.	[83]
GC-ECD		QuEChERS followed by dispersed SPE	HM	DDT, p,p'-DDE, p,p'-DDD, o,p'-DDT, p,p'-DDT, aldrin, dieldrin, endosulfan α , hexachlorobenzene	LOD: 0.018–0.078 μ g g ⁻¹ milk fat /LOQ: 0.062 to 2.38 μ g g ⁻¹ milk fat		[34]

Table 2. Cont.

Technique(s) Used [†]	MS Apparatus and/or Ionization Mode	Sample Preparation ^{††}	Matrix	OCP Used for Method Development/Application	LOD/LOQ (mg kg ^{−1})	Monitoring/Measurement in Milk Samples (Number of Samples/Number of OCPs above LOD)	Reference
GC-NCI-MS	negative chemical ionization-mass spectrometry	SE	HM	α-HCH, β-HCH, γ-HCH (lindane), δ-HCH, p,p'-DDT, o,p'-DDT, p,p'-DDE, o,p'-DDE, p,p'-DDD, o,p'-DDD, HCB, aldrin, di eldrin, endrin, trans-chlordane, trans-nonachlor, cis-nonachlor oxychlordane, heptachlor, trans-heptachlor epoxide, cis-heptachlor epoxide, mirex	LOD: 0.00 to 1660 ng g ^{−1} lipid	Twenty-four pooled samples (from 1237 milk samples)/DDTs (from 153.6 ng g ^{−1} to 1756.3 ng g ^{−1} lipid), HCHs (from 55.8 ng g ^{−1} to 536.4 ng g ^{−1} lipid) and HCB (from 18.4 ng g ^{−1} to 56.8 ng g ^{−1} lipid) detectable in every pooled sample; CHLs (from 6.1 ng g ^{−1} to 25.2 ng g ^{−1} lipid), drins (from 7.9 ng g ^{−1} to 21.8 ng g ^{−1} lipid), and mirex (from not detected to 21.8 ng g ^{−1} lipid) detected in 75.0%, 29.2% and 20.8% of samples, respectively	[95]
GC/MS and LC/MS/MS	EI/SIM mode (mainly) LC/MS: triple quadrupole using electrospray ionization.	QuEChERS and MSPD	M	Chlordane, DDE, dieldrin, endosulfan sulfate, heptachlor epoxide and lindane		(b)	[55]
GC-ECD		MSPD	UHTM	22 OCPs (HCB, α-HCH, β-HCH, γ-HCH, aldrin, dieldrin, endrin, heptachlor, heptachlor epoxide, α-chlordane, γ-chlordane, α-chlordene, trans-nonachlor, α-endosulfan, β-endosulfan, endosulfan sulfate, o,p'-DDD, p,p'-DDD, o,p'-DDE, p,p'-DDE, o,p'-DDT, p,p'-DDT	Detection limit: from 0.02 to 0.1 µg L ^{−1} /LOQs: from 0.02 µg L ^{−1} to 0.58 µg L ^{−1}	Twenty-five milk samples/HCB at 0.6 µg L ^{−1}	[104]

(a) not reported. (b) not specified. ^{††} Pretreatment methods: LLE: liquid–liquid extraction, SPE: solid-phase extraction, QuEChERS: Quick Easy Cheap Effective Rugged Safe Method, GDME: Gas-diffusion microextraction, HF-SBSE: hollow fiber–stir bar sorptive extraction, SPDE: stochastic partial differential equation, SE: soxhlet extraction, SLE: solid–liquid extraction, MSPD: matrix solid-phase dispersion. Type of milk: HM: human milk, AM: animal milk, IM: infant milk, RM: raw milk, M: milk, PM: powdered milk, UHTM: ultra-high temperature milk. [†] Techniques used: GC-MS: gas chromatography–mass spectrometry, GC-ECD: gas chromatography–electron capture detector, GC-NCI-MS: gas chromatography–negative chemical ionization–mass spectrum, HPLC-MS/MS: high-performance liquid chromatography–tandem mass spectrometry, LC-MS/MS: liquid chromatography with tandem mass spectrometry.

7. The NMR Approach—NMR and MS Metabolomics

Metabolomics, through the study of small molecules, provide information for the physiological state of a living organism [129]. Analysis of biofluids provides information for the biochemical status of an organism; the composition of biofluids can also be impor-

tant for toxicological studies and the study of metabolism, the fate of drugs, and other xenobiotics [130].

The metabolic state has been analyzed mainly through two approaches: MS and NMR. The latter, despite its lower sensitivity, offers unparalleled advantages over MS [129].

Both techniques allow the detection of hundreds of signals belonging to different metabolites in complex matrices [131] and have played a dominated role in the metabolomics studies, having complementary strengths and limitations [132].

The metabolomics' tools could provide an insight into the possible alteration of the metabolome upon possible POP exposure. Generally, the combination of several omics approaches could help the exploration of toxic mechanisms of environmental pollutants and the evaluation of their toxic mechanisms [133].

The NMR is a platform for the application of metabolomic methods, mainly through 1D ^1H NMR spectroscopy, bearing the inherent advantages of the NMR technique, among them the non-destructive nature of the measurements, the benefits of the ability to monitor a plethora of analytes in an intact sample, and the minimal—or even without any—sample pretreatment even without the need of calibration standards. Moreover, quantitative results can be obtained, and structure elucidation of unknown compounds could be performed. Through the *-omics* approach, (bio)indicator(s) could/and have been also proposed. NMR has the advantages of good stability and repeatability of the results along with the above reported—relatively simple sample pretreatment and unbiased non-destructive detection ([133] and references therein).

NMR metabolomics in food has been successfully applied in milk [22]—or through the chemometric approach [134] or milk metabolite analysis [135]—in olive oil [23,136] and is a blooming region for a range of scientific fields: from food chemistry and technology to human [137] and living organisms.

The *-omics* approach has been used in the field of food authentication [138] through HRMS and/or NMR-targeted or non-targeted approaches [139], in food safety control and food quality analyses [140], or as a tool to elucidate the biochemical responses of various environmental pollutants [141].

Direct observation of OCPs through NMR spectroscopy has not been reported up to now, although recently, the potential of NMR for chlorine contaminants in edible oils has been reported [142].

From all our previous sessions analyzed, reviewed, and commented on in our review, in Figure 3 we illustrated the holistic approach for the OCPs occurrence and impact.

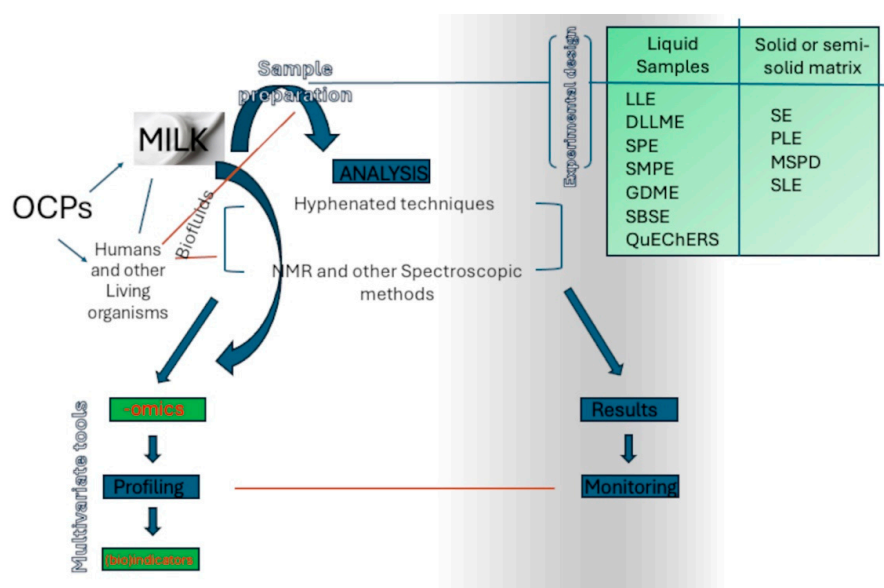


Figure 3. The holistic approach for the OCPs occurrence and their impact.

7.1. NMR-Based Metabolomics

The application of NMR-based metabolomics in the study of the effect of even the reveal of possible exposure to POPs has been proposed. A comprehensive metabolome study has confirmed the metabolic alteration among women with deep endometriosis that could be also associated with the exposure to POPs [143]. In the above study, elevated serum levels of lactate, ketone bodies, and multiple amino acids and lower levels of bile acids, phosphatidylcholines (PCs), cortisol, and hippuric acid have been reported, and the metabolite 2-hydroxybutyrate has been positively associated with endometriosis risk and exposure to *trans*-nonaol.

On the other hand, in the NMR-based metabolomics analysis of human plasma for possible exposure to POPs [27] through the metabolic profiling of 276 Korean participants, although PCBs have been associated with 3 metabolites—creatinine, acetate, and formate—the OCPs were not associated with any circulating metabolites among the 5 correlated metabolites.

The metabolic approach for food safety through NMR-based metabolomics [144] has also shown PCBs and OCPs-induced differential metabolic disturbances in fish—bluefin tuna—with alterations mainly found in energy-producing metabolic pathways, amino acid and lipid metabolism, and activation of fatty acid biosynthesis and ketogenesis.

Following the above and through the relevant limited in number—from our literature revision—from our point of view, it would be of great interest the metabolomic study of possible alterations to the milk profiling upon possible POPs exposure.

7.2. MS Metabolomics

MS metabolomics has also been applied by Yang et al. [145] in the study of maternal pesticide exposure—with OCPs among them—and birth outcomes by analyzing mother serum samples through GC-MS using a triple quadrupole mass spectrometer for the detection of the pesticides and ultra-performance liquid chromatography tandem high resolution mass spectrometry (UPLC-HRMS with a Q-Exactive mass spectrometer) for the metabolomic analysis, and the authors concluded the β -HCH—and also the organophosphate insecticide mecarbam—significant relation to a decrease in birth weight by disturbing glyceraldehyde metabolism and thyroid hormone metabolism.

Liu S et al. [146] studied the effect of polystyrene microplastics (PS MPs) and DDT on the *E. coli* microbial physiology and metabolism using an ultra-high-performance liquid chromatography electrospray ionization. Fourier-transform ion cyclotron resonance mass spectrometry (UPLC-ESI-FTICRMS), indicating the complex combined toxic effect of nanoscale plastics and the hazardous chemicals.

For other living organisms, the effect of sub-lethal exposure of deltamethrin and DDT on *Xenopus laevis* frog has been investigated recently [147] using liver metabolomics, among other techniques. Metabolome analysis was performed using GC/TOF-MS apparatus for the whole metabolome screening analysis—562 spectral compound peaks detected in the frog livers. Alanine appeared to be a suitable biomarker for insecticide exposure, and the authors also suspect the ratio between alanine and glycine may hold greater importance for insecticide exposure. Squalene was also down-regulated on DDT exposure. Nevertheless, the authors propose that in-depth targeted analysis of the metabolites of interest may be needed to provide robust biomarkers of exposure. Moreover, potential unique marker metabolites, including L-arabinopyranose and glycerol-3-phosphate for DDT exposure, were identified in their study; the general pesticide exposure markers, including L-sorbitol, myo-inositol, oleic acid, and cadaverine, possibly linked to a general stress response, were also identified.

In humans, the metabolic fingerprint of two OCPs (p,p'-DDE and HCB) exposure has been studied [148]. Non-targeted metabolomics had been performed using ultra-performance liquid chromatography coupled to time-of-flight mass spectrometry (UPLC-TOFMS) with an atmospheric electrospray interface operating in positive ion mode. The authors reported that increased exposure to p,p'-DDE was associated with decreased levels

of lysophosphatidylcholine, supporting previous experimental and epidemiological studies that suggested that endocrine-disrupting compounds, such as p,p'-DDE, might play a part in the development of various cardiometabolic diseases and that lysophosphatidylcholine metabolites could be a mediator in those events. Also, the authors observed increased exposure to p,p'-DDE association with increased levels of monoacylglycerol (18:2) and three fatty acid-related metabolites, oleamide, linolenic aldehyde, and arachidonic acid ethyl ester. Additionally, the authors reported association of increased exposure to HCB with decreased levels of two glycerophosphoethanolamine metabolites. These metabolites come from the metabolic pathways related to the metabolism of fatty acids and phosphoethanolamines.

8. Conclusions

General comments and proposals: The possible association of OCP residues' occurrence in breast milk with negative effects and/or alterations in the neurodevelopment of infants needs to be studied, and the mechanism for the development of such negative neurodevelopment should be enlightened—other persistent organic pollutants (POPs) might also affect infants' neurodevelopment in a similar manner.

Their residual analysis in milk is of major importance, and several methods have been developed and proposed. Among them, QuEChERS is the primary method of choice for several authors. PLE, whereas proposed as a promising method for complex food matrices, matrices such as those having high lipid that strongly retain analytes, have not been widely applied.

Also, up to now, there is a limited number of MSPD procedures that have been proposed for the determination of OCP occurrence in milk, although they have been applied to other fatty matrices. And SBSE is also not a widely used method for OCP determination. To the best of our knowledge, the MAE has not been applied for the extraction and determination of OCPs in milk.

The combination of metabolomics, through spectroscopic methods such as NMR complementary to MS metabolomics, for the metabolic investigation of possible alterations in human fluids combined with the quantitation data obtained through, e.g., MS techniques for possible OCP residue occurrence would also be a valuable tool in the inquiry for possible negative effects in infants or other living organisms.

For instance, we would expect that the metabolic disruptions due to possible OCP exposure might result in an alteration to the amino acid and lipid profile of the milk, and further research is needed.

In conclusion, from all the above reported in our review, OCPs occurrence in the environment and in the biosphere is an emerging trend. Along with other POPs, they pose a risk for all living organisms, and screening projects are ongoing from several organizations worldwide.

In order to determine their possible occurrence, a plethora of methods have been developed both in hyphenation and in sample preparation.

Although the usage of multivariate tools and experimental design is not also widely applied in the optimization of sample preparation, we think that for complex matrices and methods, they could be a valuable tool. Moreover, the introduction of the broader use of the *-omics* procedure to the OCPs monitoring could also be a valuable tool for their analysis and/or their effects in the living organisms.

The expansion of such an approach to the POP pollutants could contribute to our effort to study their impact and could be a challenging trend in the scientific community.

Finally, from our point of view, both the large-scale (or national) monitoring of OCPs—along with other POPs—residues in milk and training/information of the people in the agriculture sector are essential.

Author Contributions: Conceptualization, C.G.T.; methodology, E.D.T. and C.G.T.; writing—original draft preparation, E.D.T.; writing—review and editing, E.D.T. and C.G.T.; supervision, C.G.T.; All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: No new data were created or analyzed in this study.

Conflicts of Interest: The authors declare no conflicts of interest.

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