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A Validated Method for the Determination of Neonicotinoid, Pyrethroid and Organochlorine Residues in Human Milk

Niharika Anand[1] · Abhishek Kundu[2,3] · Sujata Ray[1]

Abstract

Exposure to pesticides in the environment is sensitively indicated by the concentration of these chemicals in human milk. However, to the best of our knowledge, detection methods in human milk for the relatively new class of pesticides, neonicotinoids, are yet to be validated. We developed a method of detection of neonicotinoids in human milk, together with two other classes of pesticides, pyrethroids and organochlorines. Neonicotinoids and pyrethroids are emerging pesticides that are replacing older and more persistent chemicals such as organochlorines. We optimized a procedure for extraction of these chemicals from whole milk and report our solutions to the problems of interference by co-extracted substances. The clean-up method was optimized using a minimum amount of PSA (50 mg) and $MgSO_4$ (150 mg). This was followed by GC–MS/MS analysis (for organochlorines and pyrethroids) and LC–MS/MS (for neonicotinoids). The method was validated following SANTE/11945/2015 guidelines at concentrations 10, 20 and 100 ng g^{-1}. Limits of quantification were obtained at ≤ 2 ng g^{-1} for all pesticides and lowest validated level were 10 ng g^{-1}, with measurement uncertainty between 0.47 and 2.6 ng g^{-1}. Average recovery ranged from 84 to 102% and for most compounds was found to be more satisfactory than the original QuEChERS, AOAC 2007.01 acetate buffer method and modified QuEChERS methods. The relative standard deviation was less than 16%. The method was successfully utilized for the analysis of human milk samples from Nadia, West Bengal and was found positive for organochlorines and negative for neonicotinoids and pyrethroids.

Keywords Method validation · Modified QuEChERS · Neonicotinoids · Pyrethroids · Organochlorines · Human milk

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Introduction

The general exposure of a population to pesticides is sensitively indicated by the concentration of these chemicals in human milk [1–3]. Assessing potential health risks required developing various analytical methods [4–6]. However, detection methods are yet to be validated in human milk for certain emerging classes of compounds. To the best of our knowledge, one such class of compounds is the relatively new neonicotinoids. These chemicals have been detected in bovine milk samples of skimmed, semi-skimmed and full-cream milk, milk enriched with calcium as well as powdered milk-based infant formula [7]. However, each matrix may require a separate detection method. For example, a method for skimmed bovine milk did not provide adequate recovery in the full-cream version [7]. As the matrix of human milk differs from that of bovine, methods to detect neonicotinoids in human milk require separate validation.

This study describes the development and validation of a method to detect selected neonicotinoids together with

two other classes of pesticides, pyrethroids and organochlorines, in human milk. Four neonicotinoids of the six that are registered for use in India [8] were selected for validation. The method employs a single clean-up and extraction procedure for all three classes of compounds, followed by the analysis of neonicotinoids with LC–MS/MS and of pyrethroids and organochlorines with GC–MS/MS.

In previous investigations on bovine milk, the detection of four neonicotinoids (imidacloprid, thiacloprid, acetamiprid and thiamethoxam) included solid-phase extraction by Chem Elut cartridges, high-performance liquid chromatography and diode array detection [4]. Other methods employed a pre-treatment step of dilution and centrifugation, followed by solid-phase extraction with C-18 cartridges [7]. The analyses were carried out by ultra-high performance liquid chromatography and tandem mass spectrometry. Neonicotinoids along with their metabolites and selected macrocycliclactone pesticides were also detected in organically grown and locally purchased milk, fruits and vegetables, through solid-phase extraction with C-18 cartridges followed by ultra-high performance liquid chromatography/MS/MS [9].

Pyrethroids in milk are analysed by gas chromatography and sample preparation methods include multi-walled carbon nanotubes dispersive solid-phase extraction [10], solid-phase extraction with basic alumina and C-18 cartridges in tandem [11] and liquid–liquid extraction [12]. Both pyrethroids and organochlorines have been extracted from milk via the QuEChERS method [5], which had been originally developed by Anastassiades et al. [13, 14]. This method involves microscale extraction with a small volume of acetonitrile followed by dispersive solid-phase extraction with a major reagent [5].

We modified the QuEChERS method to develop a simple and easily implemented analytical procedure to extract the neonicotinoids, pyrethroids and organochlorines. Previous applications of this method usually first extracted the lipid fraction of milk, followed by separation of lipids from pesticides with solid-phase extraction. However, these methods are often laborious, requiring soxhlet apparatus and partitioning [15–19]. One modification of the QuEChERS method reduces solvent and chemical usage by passing lipid extraction and extracting pesticides from whole milk samples. Previously, extraction from whole milk samples was reported by Luzardo et al. [6] for organochlorines, PAHs and PCBs. While the Luzardo procedure required multiple extraction steps, the method developed in this work requires a single one only. Also, when testing the Luzardo method, we found interference of fat and co-extractives in the final extract from human colostrum during the instrumental analysis. As colostrum contains more fat than mature milk samples [6], this presented a unique set of challenges that were previously unreported. Finally, the maximum residue limit for neonicotinoids (not tested by Luzardo) is low (0.01–0.05 mg kg^{-1}, as established by European legislation) and requires the development of a method allowing low detection limits.

The classes of compounds selected in this study cover a wide range of polarity, with log K_{ow} ranging from 0.8 to 6.5 and are associated with pressing environmental concerns. The pyrethroids and neonicotinoids are gradually replacing older and more persistent chemicals such as organochlorines and organophosphates [20]. Simultaneously, the older organochlorines such as DDTs and HCHs, although regulated under the Stockholm convention on persistent organic pollutants, continue to be released from diffuse sources such as old stockpiles, older functioning equipment [21] and contaminated soils, waters, vegetation and sediments. To understand and manage the impacts of these chemicals during this transition, we require a method to simultaneously monitor their environmental concentrations.

The impact of chronic pyrethroids and neonicotinoids exposure in humans over the long term is still unknown [22]. Neonicotinoids, which were detected at trace levels in the nectar and pollen of crops, are believed to be implicated in the decline of bee populations [23]. They were reported to travel up the food chain, decreasing the activity of the target pest's predators, rather than the pest itself [24]. While pyrethroids were formerly believed to be easily converted to non-toxic metabolites in the body, recent studies have reported their bio-accumulation in human milk [25]. Also, concerns were raised regarding their effects on sperm quality, reproductive hormones and pregnancy outcomes [22]. Pyrethroids were detected in urban surface waters at concentrations believed to be toxic to aquatic life [26]. Finally, organochlorine emissions, triggered by declining atmospheric concentrations, may continue for decades [27, 28].

The aim of this work was to develop a multi-residue method that allows the determination of organochlorines, pyrethroids and neonicotinoids in human milk. This procedure was successfully applied to determine the concentrations of the pesticides in human colostrum samples collected from the Nadia district in West Bengal, India.

Materials and Methods

Chemicals and Reagents

Certified reference standards of all pesticides were of > 98% purity and purchased from Sigma-Aldrich (Germany). Primary secondary amine (PSA, 40 μm, Bondesil, Agilent Technologies, USA) was used as sorbent for dispersive solid-phase extraction (d-SPE) and clean-up. The salts were dehydrated and stored in a desiccator before use.

Preparation of Standard Solutions

Stock solutions of individual pesticide standards were prepared by dissolving 10 mg ($\pm$ 0.01 mg) of each analyte in 10 mL ethyl acetate and stored at 4 °C. An intermediate stock standard mixture of 10 μg g^{-1} was prepared for organochlorines and pyrethroids by dilution in ethyl acetate and for neonicotinoids by dilution in methanol, from which calibration standards were prepared within the range 0.1–100 ng g^{-1}.

Extraction and Clean-Up

An appropriate method for multi-residue pesticide extraction in a number of food matrices is the QuEChERS method [6, 13, 14, 29–32]. Human milk was first checked for pesticide residue content with the original QuEChERS method [13, 14] and all concentrations were below the limit of detection. The procedure for extraction and clean-up is detailed in Fig. 1.

$$\% \text{ ME} = \frac{\text{Response of standard prepared in milk} - \text{Response of standard prepared in pure solvent}}{\text{Response of standard prepared in pure solvent}} \times 100$$

Analytical Methods

Analysis by GC–MS/MS

Organochlorines and pyrethroids were analysed with an Agilent model 7890A Gas Chromatograph (GC) and a 7000B Triple Quadrupole Mass Spectometer (MS) (Agilent Technologies, Palo Alto, USA). An Agilent J&W HP 5 MS (30 m × 0.25 mm i.d.; 0.25 μm film thickness) column was used for separation of analytes. The residue was estimated by triple quadrupole mass spectrometer using electron impact ionization (EI) and Multiple Reaction Monitoring (MRM) mode. The initial inlet temperature was held at 70 °C for 0.12 min; then ramped to 285 °C at a rate of 200 °C min^{-1}. The column oven temperature was initially kept constant at 70 °C for 2.5 min and ramped up five times to the final temperature of 290 °C. Sequentially the temperature ramps were: attaining 180 °C at a rate of 20 °C min^{-1}; 200 °C at 5 °C min^{-1} and held for 3 min; reaching 220 °C at 5 °C min^{-1}; 240 °C at 7 °C min^{-1} and finally reaching 290 °C at 10 °C min^{-1}. The final temperature was held for 10 min and the total run time was 32.857 min.

Analysis by LC–MS/MS

Neonicotinoids were analysed with Alliance 2695 separation module Liquid Chromatograph (LC, Waters, Milford, MA, USA) coupled with a Micromass Quattro Micro Triple Quadrupole Spectrometer (Manchester, UK) and equipped with an electrospray ionization (ESI) probe.

The LC–MS/MS analysis was done by injecting an aliquot of 20 μL on a reversed phase Symmetry C-18 column (100 mm × 2.1 mm × 5 μm, Waters, USA). The mobile phase was composed of A: water: methanol (9:1) + 5 mM ammonium acetate + 0.1% acetic acid and B: methanol: water (9:1) + 5 mM ammonium acetate + 0.1% acetic acid. The mobile phase was delivered in gradient mode and the following changes occurred: 0.0–0.6 min, 95% A was employed; from 0.6 to 2 min the mobile phase was linearly changed to 5% A and from 2 to 8 min it was put on hold, from 8 to 12 min it was brought to the initial condition and from 12 to 14 min, it was put on hold with a total runtime of 14 min. A constant flow rate of 0.3 mL min^{-1} was maintained throughout the process.

Matrix Effect

The matrix effect (ME) was evaluated in human milk by the following formula:

According to the above equation, the negative and positive value of the ME indicates the matrix-induced suppression and enhancement, respectively.

Limit of Quantification (LOQ) and Lowest Validated Level (LVL)

The limit of quantification (LOQ) is the concentration of analyte at which it is detected 95% of the time and the signal-to-noise ratio is10. The LOQs were determined by injecting standards with gradually decreasing concentration up to 0.1 ng g^{-1}. The lowest concentration of analyte in sample that was quantified with precision and accuracy was selected as the lowest validated level (LVL). Here, the LVL was the lowest validated spike level providing recovery within a range of 84–102% [33].

The method was validated through a recovery experiment in which a control sample was fortified with the mixture of pesticides at three distinct concentrations: 10, 20 and 100 ng g^{-1}, followed by re-extraction and quantification of the residues. The precision of the method, which related to its repeatability, was expressed as the relative standard deviation (RSD$_r$) of six replicate measurements at each concentration on a given day by a single analyst. The intermediate precision was expressed as the relative standard deviation (RSD$_{ip}$) of six replicate measurements at each concentration by three analysts, each on a separate day.

The measurement uncertainty (MU) was assessed according to EURACHEM Guidelines [34]. Three major
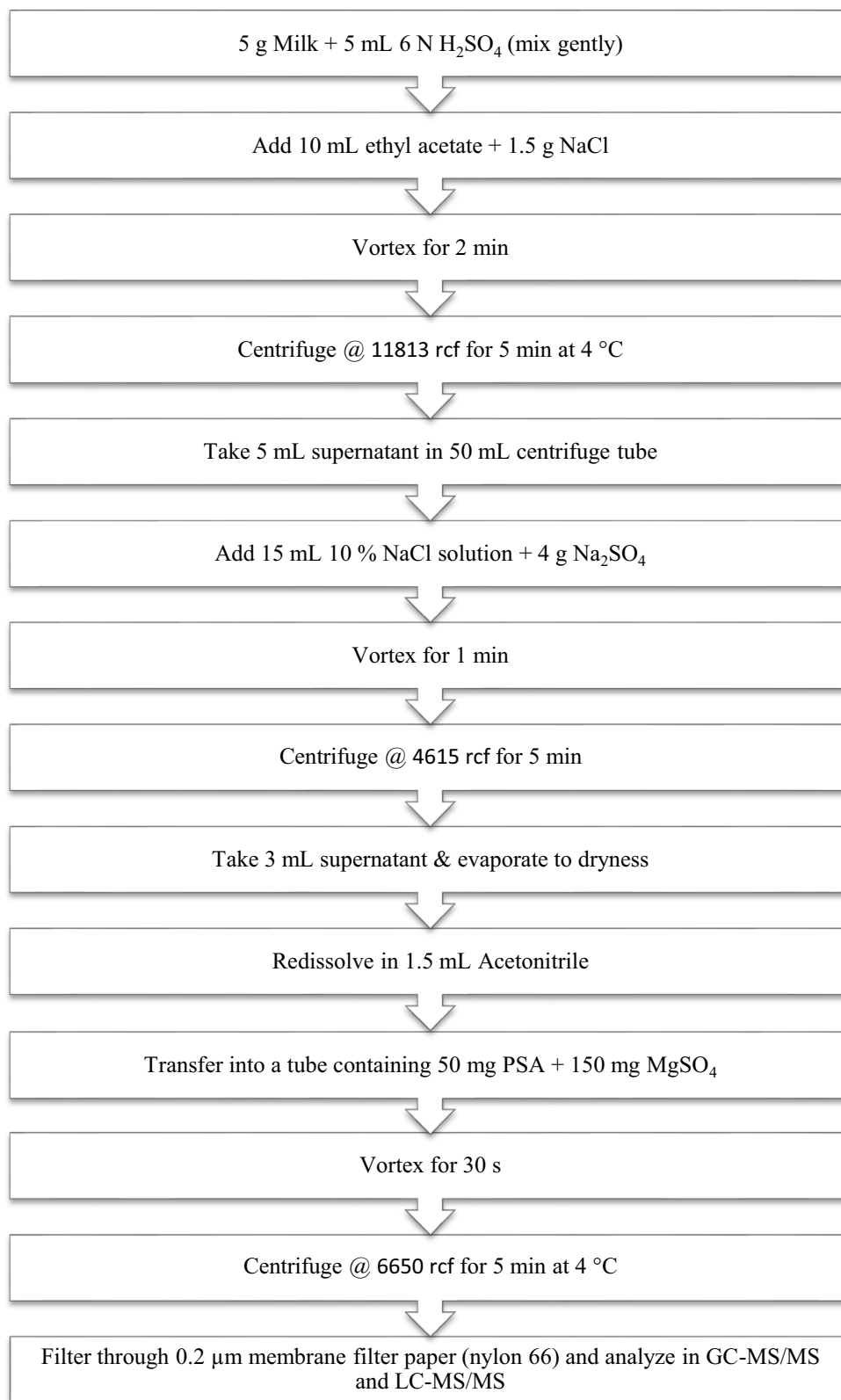
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┌─────────────────────────────────────────────────────────┐
│         5 g Milk + 5 mL 6 N H₂SO₄ (mix gently)            │
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                            ↓
┌─────────────────────────────────────────────────────────┐
│            Add 10 mL ethyl acetate + 1.5 g NaCl           │
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                            ↓
┌─────────────────────────────────────────────────────────┐
│                    Vortex for 2 min                       │
└─────────────────────────────────────────────────────────┘
                            ↓
┌─────────────────────────────────────────────────────────┐
│           Centrifuge @ 11813 rcf for 5 min at 4 °C        │
└─────────────────────────────────────────────────────────┘
                            ↓
┌─────────────────────────────────────────────────────────┐
│          Take 5 mL supernatant in 50 mL centrifuge tube   │
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│        Add 15 mL 10 % NaCl solution + 4 g Na₂SO₄          │
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│                    Vortex for 1 min                       │
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┌─────────────────────────────────────────────────────────┐
│               Centrifuge @ 4615 rcf for 5 min             │
└─────────────────────────────────────────────────────────┘
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┌─────────────────────────────────────────────────────────┐
│          Take 3 mL supernatant & evaporate to dryness     │
└─────────────────────────────────────────────────────────┘
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│              Redissolve in 1.5 mL Acetonitrile            │
└─────────────────────────────────────────────────────────┘
                            ↓
┌─────────────────────────────────────────────────────────┐
│    Transfer into a tube containing 50 mg PSA + 150 mg MgSO₄ │
└─────────────────────────────────────────────────────────┘
                            ↓
┌─────────────────────────────────────────────────────────┐
│                    Vortex for 30 s                        │
└─────────────────────────────────────────────────────────┘
                            ↓
┌─────────────────────────────────────────────────────────┐
│           Centrifuge @ 6650 rcf for 5 min at 4 °C         │
└─────────────────────────────────────────────────────────┘
                            ↓
┌─────────────────────────────────────────────────────────┐
│ Filter through 0.2 μm membrane filter paper (nylon 66) and │
│          analyze in GC-MS/MS and LC-MS/MS                 │
└─────────────────────────────────────────────────────────┘
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Flowchart steps:

1. 5 g Milk + 5 mL 6 N H_2SO_4 (mix gently)
2. Add 10 mL ethyl acetate + 1.5 g NaCl
3. Vortex for 2 min
4. Centrifuge @ 11813 rcf for 5 min at 4 °C
5. Take 5 mL supernatant in 50 mL centrifuge tube
6. Add 15 mL 10 % NaCl solution + 4 g Na_2SO_4
7. Vortex for 1 min
8. Centrifuge @ 4615 rcf for 5 min
9. Take 3 mL supernatant & evaporate to dryness
10. Redissolve in 1.5 mL Acetonitrile
11. Transfer into a tube containing 50 mg PSA + 150 mg $MgSO_4$
12. Vortex for 30 s
13. Centrifuge @ 6650 rcf for 5 min at 4 °C
14. Filter through 0.2 μm membrane filter paper (nylon 66) and analyze in GC-MS/MS and LC-MS/MS

Fig. 1 Extraction and clean-up process for pesticide residue analysis in human colostrums

sources of uncertainty, that is, the uncertainty associated with the calibration curve, repeatability and the intermediate precision were considered to calculate the Combined Standard Uncertainty. It was multiplied by a coverage factor of 2 at 95% level of confidence to obtain the Expanded Uncertainty.

The results are presented as the mean values ± standard deviation (SD). The data were analysed by one-way ANOVA followed by Duncan's test using XLSTAT (2014.5.03).

Results and Discussion

Method Development

Optimization of GC–MS/MS Analysis

Optimization of GC–MS/MS parameters was accomplished by adjusting the operating conditions of compartments such as the inlet and mass spectrometer. Each pesticide was scanned for precursor and product ions. The precursor ion was selected as that with the highest abundance after the first fragmentation. This was further fragmented using nitrogen as the collision gas, applying collision energy that ranged from 5 to 40 eV. From the scanned data, the two best transitions were selected to form the MRM (SI Tables 1.1 and 1.2). The transition with the highest response was used for quantification.

The first attempt at quantification was in cold splitless mode with 1 µL injection. However, for most compounds, this achieved a limit of detection no lower than 5 ng g^{-1}. A higher sensitivity was obtained with high volume injection using PTV solvent vent mode. A 5 µL of aliquot was injected at 70 °C. To eliminate solvent vapour, the split vent was kept open and the purge flow through it was maintained at 100 mL min^{-1} for 0.1 min. Changes in the inlet pressure

did not result in significant improvement in response and was kept at 5 psi as per the manufacturer's initial recommendation. To prevent loss of target analytes, the initial temperature of the inlet was kept constant until the split vent was closed. The non-polar stationary phase was selected as 5% phenyl dimethyl polysiloxane. Two columns, the VF-5MS and HP-5MS, were tested in identical operating conditions and HP-5MS was selected because it provided better peak separation. The total ion chromatograms (TIC) for organochlorines and pyrethroids showed clear separation of chromatographic peaks (Fig. 2). Extracted chromatogram of small peaks (allethrin, β-endosulfan and p,p-DDT) are shown in SI Figure 1.

Optimization of LC–MS/MS Analysis

Standard solutions of the four neonicotinoids (acetamiprid, imidacloprid, thiacloprid and thiamethoxam) were continuously infused into the source by an ESI probe. Full scan data were recorded in both positive and negative ionization mode. Positive ionization was found to be suitable for the neonicotinoids. The precursor ion was selected as that with the highest abundance after the first ionization. The precursor ion was further fragmented by applying varying levels of collision energy. The two most abundant ions were selected along with the precursor ion to form the MRM. Of the two transitions, that with the higher response was used for quantification and the other for confirmation of occurrence (SI Table 1.3). The capillary voltage was selected as that which resulted in the highest abundance of the precursor ion. The chromatograms for neonicotinoids showed clear separation of chromatographic peaks (SI Figure 2).

Optimization of extraction and clean-up

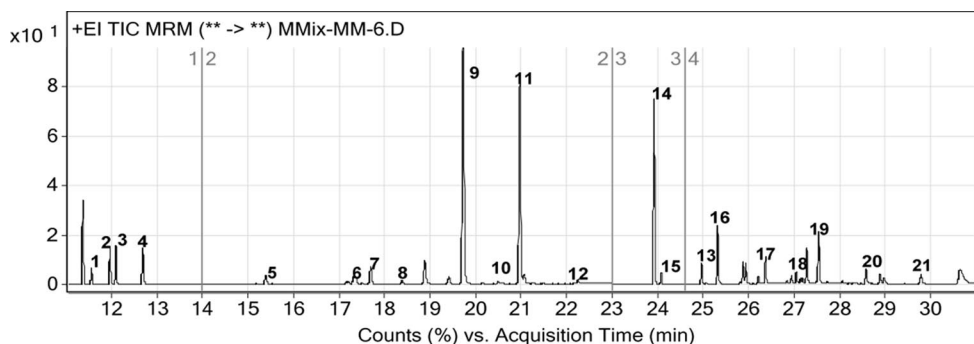Methods for extraction of organochlorine pesticides in human milk [6, 35–37] usually include several tedious

Fig. 2 Total ion chromatogram (TIC) of GC–MS/MS analysis of human milk extract spiked with 100 ng g^{-1} mixed standard (1 HCB, 2 β-HCH, 3 γ-HCH, 4 δ-HCH, 5 Aldrin, 6 Allethrin, 7 o,p-DDE, 8 α-Endosulfan, 9 o,p-DDD, 10 β-Endosulfan, 11. p,p-DDD, 12 p,p-DDT, 13 Mirex, 14 Bifenthrin, 15 Fenpropthrin, 16 λ-Cyhalothrin, 17 Permethrin,18 Cyfluthrin, 19 α-Cypermethrin, 20 Fenvalerate, 21 Deltamethrin)

steps such as classical liquid–liquid partitioning, solid-phase extraction, multiple sample extraction steps, column clean-up, as well as large quantities of solvents, sorbents and glassware. To address these problems, the goal of this study was to develop a simple, cheap but effective method that eliminated many of these steps, and was adapted for simultaneously monitoring both the older and the emerging chemicals of interest. The selection of the most suitable method of extraction was accomplished by comparing the efficiency and the recovery percentage of the following methods: (i) original QuEChERS [12, 13], (ii) AOAC acetate buffer method (AOAC official method 2007.01), (iii) QuEChERS procedure with dispersive solid-phase extraction (d-SPE) using primary secondary amines (PSA) [6] and (iv) QuEChERS extraction using ethyl acetate (instead of the acetonitrile used in original QuEChERS method) followed

by d-SPE. To eliminate the high lipid content of colostrum, the method was preceded by sulfuric acid treatment and cold centrifugation. This is the method optimized in this study. The first three methods were selected as they were successfully utilized in the extraction of pesticides from milk.

The recovery range of the optimized method was found to be satisfactory for all pesticides as compared with other tested methods (Figs. 3, 4 and SI Table 2). Average recoveries of the pesticides ranged from 84 to 102%. While using the original QuEChERS method, low average recovery for HCB (59.2%) and slightly higher than 120% for β-HCH, δ-HCH and β-endosulfan were obtained. The AOAC acetate buffer method showed satisfactory recovery for most of the compounds except hexachlorobenzene (HCB) (45.6%), mirex (65.2%) and aldrin (68%) whereas low recovery was found for pyrethroids and

Fig. 3 Comparative recoveries of organochlorines from human milk samples spiked at 100 ng g^{-1}. Four methods were tested to obtain the optimum procedure for analysis
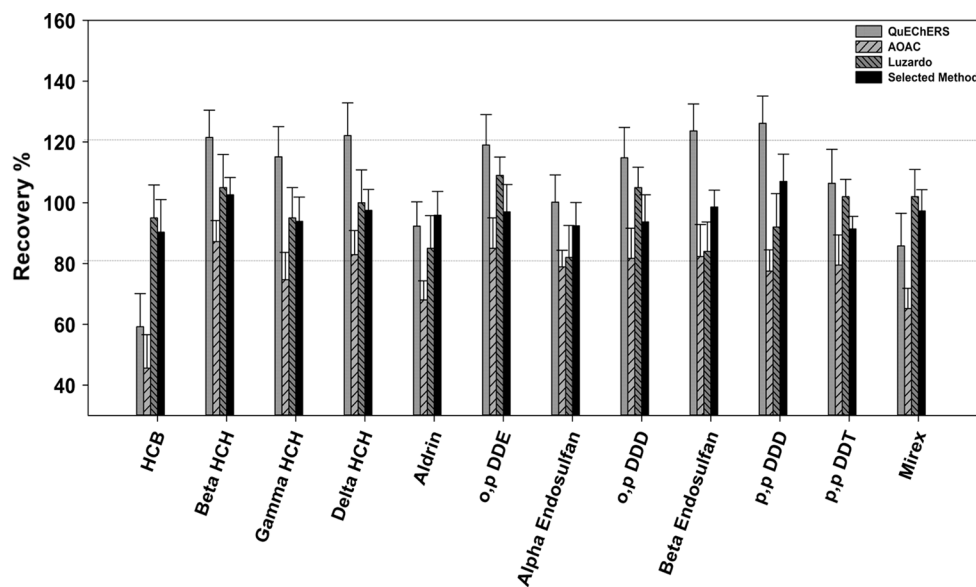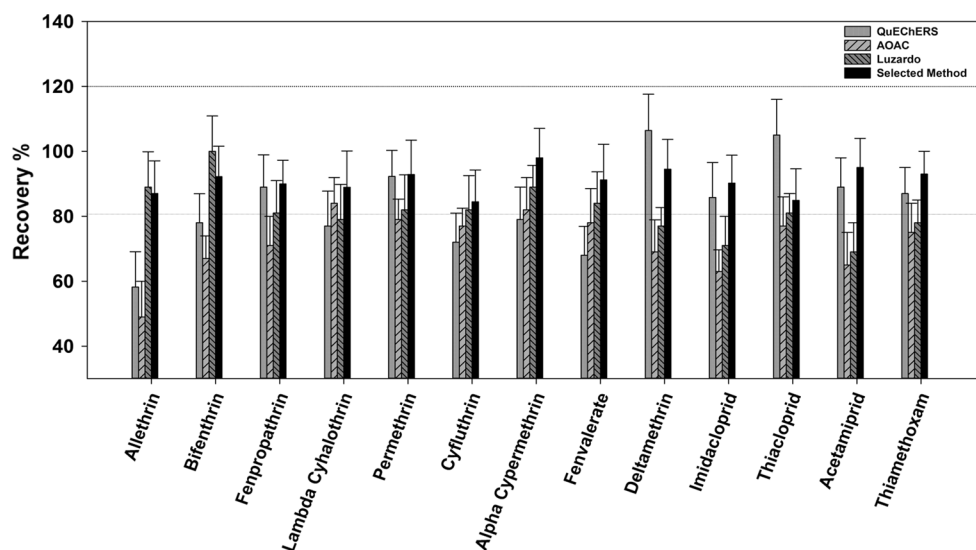

Fig. 4 Comparative recoveries of neonicotinoids and pyrethroids from human milk samples spiked at 100 ng g^{-1}

neonicotinoids. The method proposed by Luzardo et al., in which acetonitrile saturated with hexane was used instead of pure acetonitrile, provided good recoveries (> 85%) for organochlorines whereas low recovery was found for pyrethroids and neonicotinoids.

However, in this study, the previously reported methods resulted in non-reproducible retention time of the analytes. A gradual shift of retention time to the higher end was observed with increasing number of sample injections. This may be due to the deposition of matrix co-extractive in the GC inlet. Hence, a pre-treatment with sulphuric acid effectively minimized the amount of fat and proteinaceous co-extractives. In addition, cold centrifugation (at 4 °C) was also employed where most of the fats were frozen out from the extract. The sample extract in ethyl acetate was evaporated to dryness and re-constituted with acetonitrile to further reduce co-extractives dissolved in ethyl acetate.

The method reported by Luzardo et al. [6] requires extraction of the sample with the solvent mixture twice and uses two extraction solvents, acetonitrile and hexane. In the method proposed by this study, a single step extraction was accomplished using ethyl acetate alone. This reduced both the number of extraction steps as well as the number of extraction solvents. The solvent ethyl acetate has been used in the extraction of pesticides from fruit and vegetable matrixes [38, 39]. This solvent provided higher recovery of

HCB in this study compared to acetonitrile, which may be attributed to its more non-polar nature.

A dispersive solid-phase extraction (d-SPE) technique was employed to clean up the sample extract. The selection of clean-up sorbents was based on the results obtained from a comparative study in human milk, conducted with the following sorbent combinations: M1 = PSA + $MgSO_4$; M2 = PSA + $MgSO_4$ + C-18; M3 = PSA + $MgSO_4$ +C-18 + Florisil; M4 = PSA + $MgSO_4$ + Florisil (SI Table 3). A comparison of the results is shown in Figs. 5a, b and SI Table 4. Statistical significance testing confirms that the addition of C-18 and/or florisil does not improve the recovery of most pesticides. Therefore, the extract was cleaned up using method M1, that is, d-SPE using the minimum required amount of PSA (50 mg) and $MgSO_4$ (150 mg).

Method Validation

The method was validated in human colostrums following European Union guidelines SANTE/11945/2015 [40] and IUPAC guidelines for single laboratory validation. The presence of target compounds was confirmed by comparing the ion ratios in the spiked sample with that of matrix-matched calibration standards. A positive matrix influence was observed for all compounds and was higher in case of pyrethroids than organochlorines. To minimize the effect of
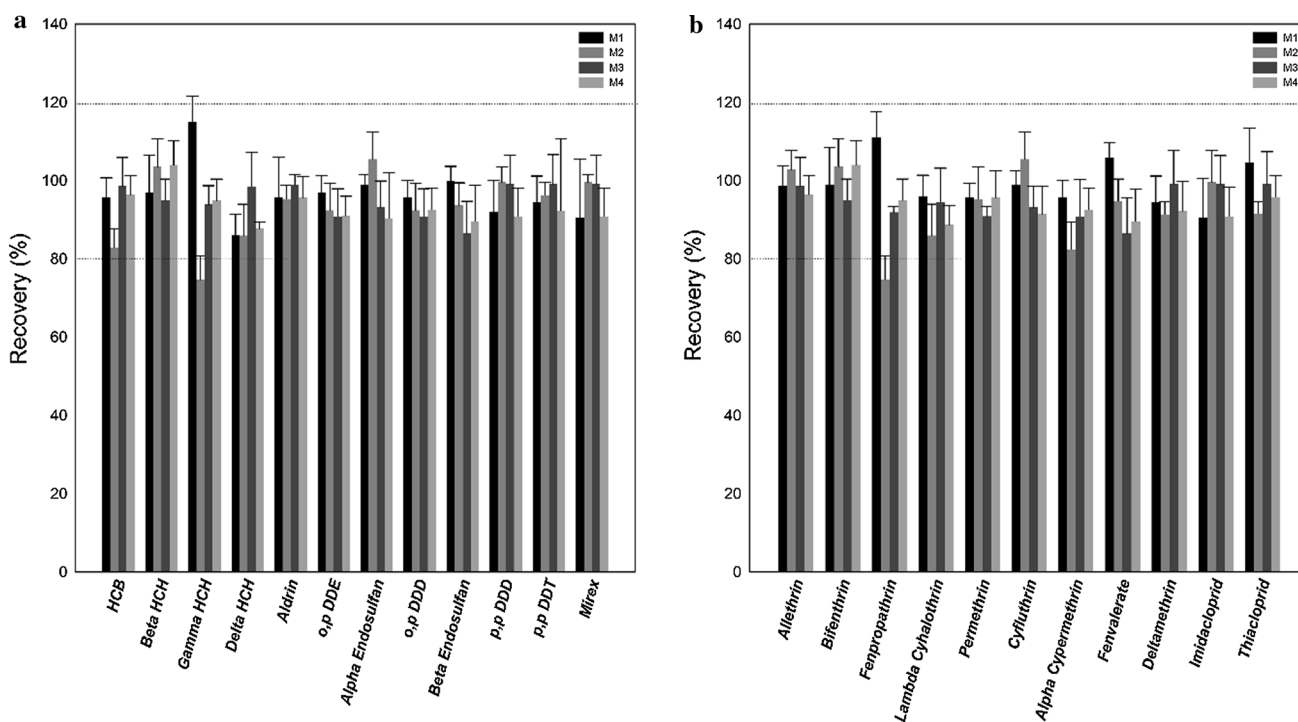

Fig. 5 Recovery (%) obtained from the clean-up experiments on human milk for organochlorines (**a**) and pyrethroids and selected neonicotinoids (**b**) (where, M1 = PSA + $MgSO_4$, M2 = PSA + $MgSO_4$ + C-18, M3 = PSA + $MgSO_4$ + C-18 + Florisil, M4 = PSA + $MgSO_4$ + Florisil)

Table 1 Validation parameters obtained for the optimized method in human milk: limit of quantification (LOQ), lowest validated level (LVL), average recovery (%), intermediate precision (IP) measured by relative standard deviation (% RSD) and measurement uncertainty (MU)

Compound name	LOQ (ng g^{-1})	LVL (ng g^{-1})	% Average recovery (% RSD)			MU @ LVL
			10 ng g^{-1}	20 ng g^{-1}	100 ng g^{-1}	10 ng g^{-1}
HCB	0.1	10	90 (6)	91 (10)	88 (9)	0.47
β-HCH	0.5	10	103 (8)	104 (9)	100 (9)	0.54
γ-HCH	0.5	10	94 (10)	93 (9)	93 (7)	0.89
δ-HCH	0.5	10	96 (10)	92 (9)	96 (10)	1.32
Aldrin	0.2	10	93 (8)	96 (8)	93 (7)	0.66
Allethrin	0.5	10	90 (12)	92 (9)	87 (10)	1.07
o,p-DDE	0.1	10	91 (11)	93 (9)	86 (8)	0.95
α-Endosulfan	0.5	10	92 (10)	98 (10)	93 (7)	0.78
o,p-DDD	0.1	10	89 (12)	94 (11)	92 (11)	0.97
β-Endosulfan	1.0	10	95 (10)	98 (8)	97 (8)	0.93
p,p-DDD	0.2	10	92 (10)	97 (10)	90 (8)	0.81
p,p-DDT	0.5	10	92 (10)	92 (9)	97 (7)	1.18
Bifenthrin	0.1	10	91 (7)	87 (11)	92 (9)	0.53
Fenpropathrin	1.0	10	91 (6)	89 (9)	90 (7)	0.61
Mirex	0.5	10	93 (9)	96 (12)	94 (9)	0.67
λ-Cyhalothrin	1.0	10	91 (6)	90 (14)	89 (11)	0.47
Permethrin	1.0	10	93 (9)	95 (8)	93 (11)	0.66
Cyfluthrin	2.0	10	88 (13)	89 (16)	84 (10)	1.28
α-Cypermethrin	2.0	10	96 (11)	102 (9)	98 (9)	1.10
Fenvalerate	2.0	10	89 (11)	94 (13)	91 (11)	1.12
Deltamethrin	2.0	10	96 (12)	92 (11)	94 (9)	1.38
Thiacloprid	2.0	10	88 (7)	87 (8)	85 (10)	1.45
Imidacloprid	1.0	10	93 (9)	89 (10)	90 (9)	2.61
Acetamiprid	1.0	10	95(10)	98 (8.3)	95(9)	0.95
Thiamethoxam	1.0	10	93(8)	92(10)	93(7)	1.05

matrix influence, matrix-matched calibration curves were used. Acceptable validation criteria for calibration curves were a regression coefficient greater than 0.99.

The LVLs for all pesticides were determined at the level of 10 ng g^{-1} (Table 1). For neonicotinoids, this was below the low maximum residue limit established by European legislation (0.01–0.05 mg kg^{-1}). The LOQs for organochlorines were lower compared to those of the pyrethroids and neonicotinoids. The average recoveries (Table 1) of all analytes were within acceptable range (70–120%). Repeatability for most compounds in terms of % RSD$_r$, estimated from single day results ($n = 6$), were < 10%. Intermediate precision (% RSD$_{ip}$) as a measure of intra laboratory variation was also within acceptable range, i.e. below 20% (Table 1). Estimated values of Measurement Uncertainty (MU) as shown in Table 1 were in the range of 0.47–2.61 ng g^{-1} (SI Table 5) which satisfies the validation criteria.

Colostrum Analysis

The validated method was applied to detect and quantify the pesticide residues in ten human milk samples collected from hospitals in Nadia, West Bengal. As the samples were collected within 7 days after birth, they contained pure colostrum (SI Table 6). It was found that most samples contained detectable levels of DDT and its metabolites and HCHs (Table 2, Fig. 6). This implied that the general population in the area studied is widely exposed to these contaminants. The remaining organochlorine pesticides were either absent

Table 2 Concentration of pesticide residues (ng g^{-1}) found in human milk samples collected from Nadia district, West Bengal

Compound	NA1	NA2	NA3	NA4	NA5	NA6	NA7	NA8	NA9	NA10
p,p-DDD	‹LOQ	‹LOQ	‹LOQ	12.19	‹LOQ	‹LOQ	‹LOQ	‹LOQ	‹LOQ	‹LOQ
p,p-DDT	‹LOQ	31.31	‹LOQ	‹LOQ	16.13	13.78	61.57	–	13.83	16.69
β-HCH	‹LOQ	12.07	–	‹LOQ	‹LOQ	‹LOQ	‹LOQ	–	–	‹LOQ
γ-HCH	‹LOQ	22.07	–	‹LOQ	‹LOQ	–	‹LOQ	–	‹LOQ	‹LOQ

Fig. 6 GC–MS/MS extracted chromatograms of real milk samples showing quantification transitions of *p,p*-DDT containing **a** *p,p*-DDT at 31.31 ng g^{-1}, **b** *p,p*-DDT at 16.13 ng g^{-1}, **c** *p,p*-DDT at 13.78 ng g^{-1}, **d** *p,p*-DDT at 61.57 ng g^{-1}, **e** *p,p*-DDT at 13.83 ng g^{-1}, **f** *p,p*-DDT at 16.6 ng g^{-1}
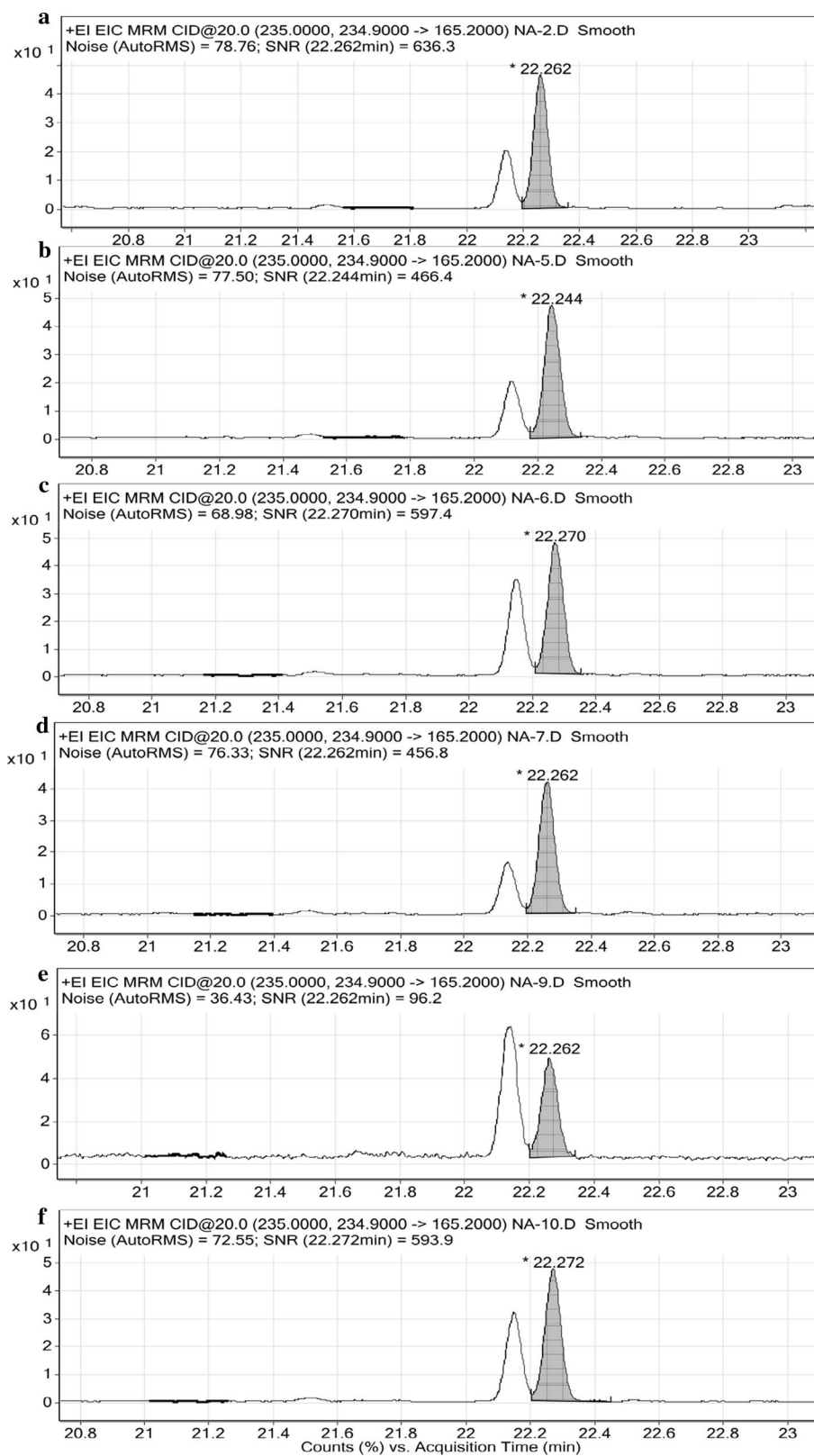
or present at levels below the detection limits. However, no detectable quantity of neonicotinoids and pyrethroids was detected. The obtained results are in good agreement with the previous literature reports [16, 17].

Conclusion

A simple, rapid, operator friendly as well as economic method was developed and validated. It provides simultaneous determination of organochlorines, pyrethroids and neonicotinoids in a single extraction step through a modification of the QuEChERS method and GC–MS/MS and LC–MS/MS analysis. To the best of our knowledge, this is the first validation of a method to detect neonicotinoids in human milk. The selected method for d-SPE is more promising than those reported previously ensuring satisfactory precision and accuracy. This approach effectively eliminates fat and co-extractives from the sample extracts. Above all, the method significantly reduces sample and solvent volume. It was successfully applied to human colostrum samples. These samples contained DDTs and its metabolites and HCHs, indicating that the general population is exposed to pesticide contamination in the area.

Compliance with ethical standards

Ethics approval The study was approved by the ethics committee of Indian Institute of Science Education and Research (IISER), Kolkata. All study participants were informed of the purpose and procedures before the study, and written consent were obtained.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. WHO (2009) Biomonitoring of human milk for persistent organic pollutants (POPs). http://www.who.int/foodsafety/chem/pops_biomonitoring/en/. Accessed 15 Jan 2017
2. Çok I, Mazmanci B, Mazmanci MA, Turgut C, Henkelmann B, Schramm KW (2012) Analysis of human milk to assess exposure to PAHs, PCBs and organochlorine pesticides in the vicinity Mediterranean city Mersin, Turkey. Environ Int 40:63–69
3. Wang DC, Yu P, Zhang Y, Cui Y, Sun CH (2008) The determination of persistent organic pollutants (POPs) in the colostrums of women in preterm labor. Clin Chim Acta 397(1):18–21
4. Seccia S, Fidente P, Montesano D, Morrica P (2008) Determination of neonicotinoid insecticides residues in bovine milk samples by solid-phase extraction clean-up and liquid chromatography with diode-array detection. J Chromatogr A 1214(1):115–120
5. Jeong IS, Kwak BM, Ahn JH, Jeong SH (2012) Determination of pesticide residues in milk using a QuEChERS-based method developed by response surface methodology. Food Chem 133(2):473–481
6. Luzardo OP, Ruiz-Suárez N, Almeida-González M, Henríquez-Hernández LA, Zumbado M, Boada LD (2013) Multi-residue method for the determination of 57 persistent organic pollutants in human milk and colostrum using a QuEChERS-based extraction procedure. Anal Bioanal Chem 405(29):9523–9536
7. Aguilera-Luiz MM, Plaza-Bolaños P, Romero-González R, Vidal JM, Frenich AG (2011) Comparison of the efficiency of different extraction methods for the simultaneous determination of mycotoxins and pesticides in milk samples by ultra high-performance liquid chromatography-tandem mass spectrometry. Anal Bioanal Chem 399(8):2863–2875
8. CIBRC (2017) CIBRC (Central Insecticide Board and Registration Committee) Pesticides Registered under section 9(3) of the Insecticides Act, 1968 for use in the Country. http://cibrc.nic.in. Accessed 10 Oct 2017
9. Kamel A, Qian Y, Kolbe E, Stafford C (2010) Development and validation of a multiresidue method for the determination of neonicotinoid and macrocyclic lactone pesticide residues in milk, fruits, and vegetables by ultra-performance liquid chromatography/MS/MS. J AOAC Int 93(2):389–399
10. Gao YL, Sun P (2017) Determination of five pyrethroid pesticides residue in liquid milk by gas chromatography using multi-walled carbon nanotubes as dispersion solid phase extraction sorbent. Acta Chromatogr 1508:1–6
11. Dallegrave A, Pizzolato TM, Barreto F, Eljarrat E, Barceló D (2016) Methodology for trace analysis of 17 pyrethroids and chlorpyrifos in foodstuff by gas chromatography–tandem mass spectrometry. Anal Bioanal Chem 408(27):7689–7697
12. Khay S, Abd El-Aty AM, Choi JH, Shin EH, Shin HC, Kim JS, Shim JH (2009) Simultaneous determination of pyrethroids from pesticide residues in porcine muscle and pasteurized milk using GC. J Sep Sci 32(2):244–251
13. Anastassiades M, Lehotay SJ, Štajnbaher D, Schenck FJ (2003) Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "dispersive solid-phase extraction" for the determination of pesticide residues in produce. J AOAC Int 86(2):412–431
14. Anastassiades M, Maštovská K, Lehotay SJ (2003) Evaluation of analyte protectants to improve gas chromatographic analysis of pesticides. J Chromatogr A 1015(1):163–184
15. Kunisue T, Someya M, Kayama F, Jin Y, Tanabe S (2004) Persistent organochlorines in human breast milk collected from primiparae in Dalian and Shenyang, China. Environ Pollut 131(3):381–392
16. Subramanian A, Ohtake M, Kunisue T, Tanabe S (2007) High levels of organochlorines in mothers' milk from Chennai (Madras) city, India. Chemosphere 68(5):928–939
17. Someya M, Ohtake M, Kunisue T, Subramanian A, Takahashi S, Chakraborty P, Tanabe S (2010) Persistent organic pollutants in breast milk of mothers residing around an open dumping site in Kolkata, India: specific dioxin-like PCB levels and fish as a potential source. Environ Int 36(1):27–35
18. Anda EE, Nieboer E, Dudarev AA, Sandanger TM, Odland JØ (2007) Intra-and intercompartmental associations between levels of organochlorines in maternal plasma, cord plasma and breast milk, and lead and cadmium in whole blood, for indigenous peoples of Chukotka, Russia. J Environ Monit 9(8):884–893
19. Bergkvist C, Aune M, Nilsson I, Sandanger TM, Hamadani JD, Tofail F, Vahter M (2012) Occurrence and levels of

organochlorine compounds in human breast milk in Bangladesh. Chemosphere 88(7):784–790

20. Trunnelle KJ, Bennett DH, Tulve NS, Clifton MS, Davis MD, Calafat AM, Hertz-Picciotto I (2014) Urinary pyrethroid and chlorpyrifos metabolite concentrations in Northern California families and their relationship to indoor residential insecticide levels, part of the study of use of products and exposure related behavior (SUPERB). Environ Sci Technol 48(3):1931–1939

21. Breivik K, Sweetman A, Pacyna JM, Jones KC (2002) Towards a global historical emission inventory for selected PCB congeners—a mass balance approach: 2. Emiss Sci Total Environ 290(1):199–224

22. Saillenfait AM, Ndiaye D, Sabaté JP (2015) Pyrethroids: exposure and health effects—an update. Int J Hyg Environ Health 218(3):281–292

23. Whitehorn PR, O'Connor S, Wackers FL, Goulson D (2012) Neonicotinoid pesticide reduces bumble bee colony growth and queen production. Science 336(6079):351–352

24. Douglas MR, Rohr JR, Tooker JF (2015) EDITOR'S CHOICE: neonicotinoid insecticide travels through a soil food chain, disrupting biological control of non-target pests and decreasing soya bean yield. J Appl Ecol 52(1):250–260

25. Corcellas C, Feo ML, Torres JP, Malm O, Ocampo-Duque W, Eljarrat E, Barceló D (2012) Pyrethroids in human breast milk: occurrence and nursing daily intake estimation. Environ Int 47:17–22

26. Kuivila KM, Hladik ML, Ingersoll CG, Kemble NE, Moran PW, Calhoun DL, Gilliom RJ (2012) Occurrence and potential sources of pyrethroid insecticides in stream sediments from seven US metropolitan areas. Environ Sci Technol 46(8):4297–4303

27. Nizzetto L, Macleod M, Borgå K, Cabrerizo A, Dachs J, Guardo AD, Ludwig B (2010) Past, present, and future controls on levels of persistent organic pollutants in the global environment. Environ Sci Technol 44(17):6526–6531

28. Evenset A, Carroll J, Christensen GN, Kallenborn R, Gregor D, Gabrielsen GW (2007) Seabird guano is an efficient conveyer of persistent organic pollutants (POPs) to Arctic lake ecosystems. Environ Sci Technol 41(4):1173–1179

29. Lehotay SJ, Maštovská K, Lightfield AR (2005) Use of buffering and other means to improve results of problematic pesticides in a fast and easy method for residue analysis of fruits and vegetables. J AOAC Int 88(2):615–629

30. Lehotay SJ, Tully J, Garca AV, Contreras M, Mol H, Heinke V, Poulsen ME (2007) Determination of pesticide residues in foods by acetonitrile extraction and partitioning with magnesium sulfate: collaborative study. J AOAC Int 90(2):485–520

31. Madureira FD, da Silva Oliveira FA, de Souza WR, Pontelo AP, de Oliveira MLG, Silva G (2012) A multi-residue method for the determination of 90 pesticides in matrices with a high water content by LC–MS/MS without clean-up. Food Addit Contam Part A 29(4):665–678

32. de Oliveira MLG, Madureira FD, Aurélio F, Pontelo AP, Silva G, Oliveira R, Paes C (2012) A multi-residue method for the determination of pesticides in high water content matrices by gas chromatography–single quadrupole mass spectrometry with electron ionisation (EI-GC/MS). Food Addit Contam Part A 29(4):657–664

33. Carneiro RP, Oliveira FA, Madureira FD, Silva G, de Souza WR, Lopes RP (2013) Development and method validation for determination of 128 pesticides in bananas by modified QuEChERS and UHPLC–MS/MS analysis. Food Control 33(2):413–423

34. Ellison SL, Rosslein M, Williams A (eds) (2012) Eurachem/CITAC guide: quantifying uncertainty in analytical measurement. 3rd edn. ISBN 978-0-948926-30-3. http://www.eurachem.org. Accessed 16 Oct 2017

35. Devanathan G, Subramanian A, Someya M, Sudaryanto A, Isobe T, Takahashi S, Tanabe S (2009) Persistent organochlorines in human breast milk from major metropolitan cities in India. Environ Pollut 157(1):148–154

36. Lacayo Romero ML, Dorea JG, Cruz Granja AC (2000) Concentrations of organochlorine pesticides in milk of Nicaraguan mothers. Arch Environ Health Int J 55(4):274–278

37. Sharma A, Gill JPS, Bedi JS, Pooni PA (2014) Monitoring of pesticide residues in human breast milk from Punjab, India and its correlation with health associated parameters. Bull Environ Contam Toxicol 93(4):465–471

38. Aysal P, Ambrus A, Lehotay SJ, Cannavan A (2007) Validation of an efficient method for the determination of pesticide residues in fruits and vegetables using ethyl acetate for extraction. J Environ Sci Health Part B 42(5):481–490

39. Banerjee K, Oulkar DP, Dasgupta S, Patil SB, Patil SH, Savant R, Adsule PG (2007) Validation and uncertainty analysis of a multi-residue method for pesticides in grapes using ethyl acetate extraction and liquid chromatography–tandem mass spectrometry. J Chromatogr A 1173(1):98–109

40. SANTE/11945/2015 (2016) Guidance document on analytical quality control and method validation procedures for pesticide residues analysis in food and feed. European Commission Health and Consumer Protection Directorate General. https://ec.europa.eu/food/sites/food/files/plant/docs/pesticides_mrl_guidelines_wrkdoc_11945.pdf. Accessed 11 Oct 2017