

Determination of Recent Concentration of DDT and its Metabolites in Breast Milk Using Various Sample Preparation Methods

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Abstract

DDT is the pesticide, which was applied in biggest amount in world wide. It has persistent, bioaccumulation and bio magnification features. The DDT is an excellent pesticide, but it shows carcinogenic, neurotoxic and strong estrogenic effects. This is the reason why its agrochemical use was banned during the late sixties. Its use, however, is allowed in the malaria epidemic cases. In this paper, the DDT and its metabolite contents of human breast milk have been investigated. In the most cases the DDE was recognized in the recent samples. Various extraction methods were compared. A modified QuEChERS method was also tested. Some tendencies were established for the DDE contents of breast milk samples.

Key words: DDT and its metabolites, breast milk, QuEChERS, GC/ECD

Introduction

The worldwide production amount of DDT exceeds the 2.6 million tons, which is the largest quantity among the pesticides (Wei et al., 2007). In Hungary, approximately 40 000 tons of DDT was used between 1945-1966 period (Lotz, 2007). Initially, DDT looked like to be a very effective pesticide and harmless for warm blooded animals. Later the several pests became resistance against DDT, and serious unwanted side effects emerged for fishes and warm blooded animals (Carlson, 1962; ATSDR, 2002). The DDT has tumorigenic, neutotoxic, estrogenic endocrin disruptive effects. One of the most shocking symptoms was the egg thinning of predatory birds, which was the reason for drastic decline of predatory birds in several areas (Lundholm, 1997). Not only the DDT, but their main metabolites show the toxic effects. DDE proved more toxic than DDT in several cases (Lundholm, 1997). Moreover the DDT and its metabolites have high persistency (Mikes et al., 2012). The DDT has more than 15 year's half-life in soil. DDE shows even higher persistency than DDT (Torres- Dosal et al. 2012; Mikes et al., 2012). The high DDE/DDT ratio show old pollution, but low DDE/DDT ratio is evidence of recent DDT pollution. The enantiomer ratios of o,p-DDE give information of origin of pollution (Bidleman et al., 2012) Other unwanted feature of DDT and its metabolites are their bioaccumulation features (Wang and Needham, 2007). Bioaccumulation means the lipophilic DDT and its metabolite deposits in adipose tissues and they can be hardly mobilized. On the other hand, they can easily mobilize by breast milk, because the milking metabolism different from normal excretion (Wang and Neddham, 2007; Azeredo et al. 2008; Mikes et al., 2012; Guerranti et al., 2011). The biomagification makes the troubles even worse, because the levels of pollutions multiply along the food chain (Wang and Needham, 2007; Mikes et al., 2012).

The persistency, bioaccumulation and biomagnification features of DDT and its metabolites can cause even hundred million higher concentrations of these compounds in the human breast milk, than their background level. Namely the infant can be expected as top predator, having increased exposure in milk. The previously mentioned unwanted effects of DDT and its metabolites resulted in the ban of the use of DDT for agricultural use in the vast majority of countries. It is worthy to note that, Hungary was the first country, which withdraws DDT from agricultural use in 1966. The DDT is also in the list of dirty dozen according to Stockholm convention (United Nations Stockholm Convention on Persistent Organic Pollutants list, 2001). The national and international campaigns were launched for the tests of DDT and its metabolites content of mother milk (WHO, 2007). These campaigns are mainly justified of the followings:

- The breast feeding period is very sensitive period for health of infants.
- The DDT and its metabolites are much higher level in breast milk than background concentrations, caused by their persistency, bioaccumulation and biomagnification features.
- The analysis methods of DDT are well established, and not so expensive.

On the other hand, the DDT is the most effective pesticide against the malaria mosquitos. Therefore the use of DDT is allowed for human health purposes in the case of prevention of malaria diseases (United Nations Stockholm Convention on Persistent Organic Pollutants list, 2001; Lotz, 2007; Azeredo et al., 200; Torres-Dosal et al., 2012). The global warming results in an estimated threat of malaria cases in Europe too (Rogers and Randolph, 2000). The Danube Delta is one of the areas; which may be affected by malaria infection in the future.

The analysis procedures require multi steps sample treatments, and dedicated instrumental analysis (EN 15281-4:1998; DFG S19: §35 LMBG 00.00.34 (2010)). The sample treatment significantly depends on the matrices. Aqueous samples can be extracted at neutral pH with methylene chloride using either EPA Method 3510 (separatory funnel), EPA-Method 3520 (continuous liquid-liquid extractor) or EPA-Method 3535 (solid-phase extraction). Solid samples may be extracted with hexane-acetone (1:1) or methylene chloride-acetone (1:1) using EPA-Method 3540 (Soxhlet), EPA-Method 3541 (automated Soxhlet), EPA-Method 3545 (pressurized fluid extraction), EPA-Method 3546 (microwave extraction), EPA-Method 3550 (ultrasonic extraction), EPA-Method 3562 (supercritical fluid extraction). The extracted samples have to be clean up before the instrumental analysis. A variety of the applied cleanup steps also depends on the nature of the matrix interferences. The applied cleanups use various chromatographic methods including EPA-Method 3610 (alumina column), EPA-Method 3620 (Florisil column), EPA-Method 3630 (silica gel column), EPA-Method 3640 (gel permeation column).

Recently the universal QuChERS (Quick, Easy, Cheap, Effective, Rugged, Safe) methods has become more and more popular (Anastassiades, 2003). These multiresidue methods are really very fast, easily managed, and having low solvent consumptions. These methods base on acetonitrile extraction, and centrifugation followed by solid phase extraction (SPE). Frequently the SPE step is made with dispersive solid-phase extraction. This method was invented for vegetables and fruits. The modified QuChERS method, however, can be applied for samples having high lipid content too (Samanidou 2008; Zhao, 2011; Misselwitz, 2011). In these cases a fat removing step is advised. On the other hand, the QuChERS method has not yet applied frequently for breast milk samples (Misselwitz, 2012). GC/ECD or GC/MS methods are appropriate for instrumental analysis (EPA-method 8081B, 2000; EN 1528, 1998; WHO, 2007). Two columns analysis methods confirm the compounds identifications in the samples. The parallel joint columns produce two retention times, and the same peak areas for one compound, which make the sample identification sure.

Our aims with this report are the following:

- To show the recent presence of DDT and its metabolites in breast milk samples in Hungary,
- To see the tendency of concentration of the DDT and its metabolites in time scale and other parameters,
- To prove the applicability of the QuChERS method for breast milk samples.

The possible threat of malaria in Europe shows that, the DDT is not an outdated analysis task even in Hungary. Moreover, the bleed of illegal hidden depots can cause recent DDT pollutions too.

Experimental

Materials and instrumentation

Method A: EN 1528-2:1998. (point 6.1.4 extraction by cooled centrifugation): residue grade solvents (acetonitrile, dichloromethane, hexane, acetone), (anhydrous Na₂SO₄ were purchased from Sigma Aldrich. Method B: EN 15662:2009 QuChERS method: QuChERS extraction kit (P/ 5982-5650) contains salts (4 g MgSO₄; 1 g NaCl; 1 g NaCitrate; 0.5 g disodium citrate sesquihydrate), QuChERS dispersive kit for SPE (P/N 5982-0028 2 ml Eppendorf tube contains 50 mg PSA, 50 mg C₁₈, 7.5 mg GCB, 150 mg MgSO₄). Organochlorine Pesticide Mixture from the Restek (Cat. no. 32415) and PCB 209 from the Riedel-de Haën (Cat No. 35587) were chosen as reference and surrogate standard respectively. The cooled (-10°C) and room temperature centrifugations were done in a laboratory centrifuge (Hermle) at 3000 rpm. An Agilent

Technologies 6890N Network GC System with HP7683 automatic injector was used for dual GC/ECD analysis. Column pair was mounted in a press-fit Y-shaped glass 3-way union splitter. Column A: Stx-CLPesticides from the Restek (30 m x 0.25 mm x 0.20 μ m, Cat No. 11543) and Column B: Stx-CLPesticides2 from the Restek (30 m x 0.25 mm x 0.20 μ m, Cat. No. 11443) were applied.

Procedures

Collection of breast milk samples (Sampling and sample preparation of breast milk samples)

7 breast milk samples were collected. The ages of mothers varied between 20-30 years. Each mother had their first delivery, because it is expected that the DDT and its metabolite have the highest content in the case of first delivery (Wang, 2007). The samples were collected in the second week after they gave birth. The milk samples were stored in deep freezer until the extractions.

The frozen samples were let to thaw and then the liquids were homogenized. 10 and 25-50 g of milk portions were subsampled in case of the QuEChERS method and the EN 1528 method respectively, and 1 μ g PCB 209 surrogate standard solutions were added. The reference sample was 25 ml cow milk which was spiked at 10 μ g/kg level with the organochlorine pesticide mixture containing the DDT and its metabolites.

Sample processing using cooled centrifugation

The samples were centrifuged for 10 minutes at 2500 rpm at 5°C. The solid fat phases were decanted and dissolved in 30 ml hexane and finally dried with anhydrous Na₂SO₄. The hexane was evaporated and the weights of the remaining fats were measured.

0.5 grams of fats were melted on hot water baths, and mixed with 3 ml extraction solutions (ACN/CH₂Cl₂- 3:1) and centrifuged (3000 rpm) for 20 min. at -10 °C. The upper layers were decanted and collected in test tubes. The extractions of fats were repeated and the two extracts were unified. The organic phases were evaporated under gentle stream of N₂ and the final volumes were adjusted with hexane to 1 ml. These solutions were taken into GC vials and analysed by GC-dual ECD..

Sample processing with the QuEChERS method

10 ml of acetonitrile were added to homogenized 10 ml liquid milk samples. 6.5 g of salt mixtures were poured into the tubes and vigorously shaken with vortex for 2 min. These were followed centrifugation (4000-4500 rpm) for 15 min. The solutions were decanted into test tubes and kept in deep freezer for overnight in order to remove the major part of fat. 1 ml of "fat-free" acetonitrile solution was further cleaned-up by the QuEChERS dispersive kit (2 ml Eppendorf tube contains 50 mg PSA, 50 mg C₁₈, 7.5 mg GCB, 150 mg MgSO₄. These materials were homogenized with 1-2 min intensive shaking. Finally the solutions were centrifuged with 4000-4500 rpm for 2 minutes. An aliquot was taken out and evaporated to dryness at 35°C using a gentle stream of nitrogen. Then it was dissolved in 1 ml hexane and taken into GC vials and analysed by GC-dual ECD.

The GC measurements had the following temperature program: 90°C (1.6 min), 50 °C/min 170°C, 3.5°C/min 300°C. The injection modes were splitless 1 μ l (1 min) at 270°C. H₂ was the carrier gas with 13.01 psi inlet pressure. Detectors were μ ECDs at 330°C using N₂ make-up gas. The chromatograms were treated with ChemStation software (Agilent). Calibration curves are built/drawn in 0.2 -50 μ g/l concentration range of DDE. The advantage of using dual detection system is that we can choose one channel for the quantitative analysis and the other is suitable for qualification.

Results and discussions

The chromatograms were appropriate to determine the compound of interests (Figure 1).

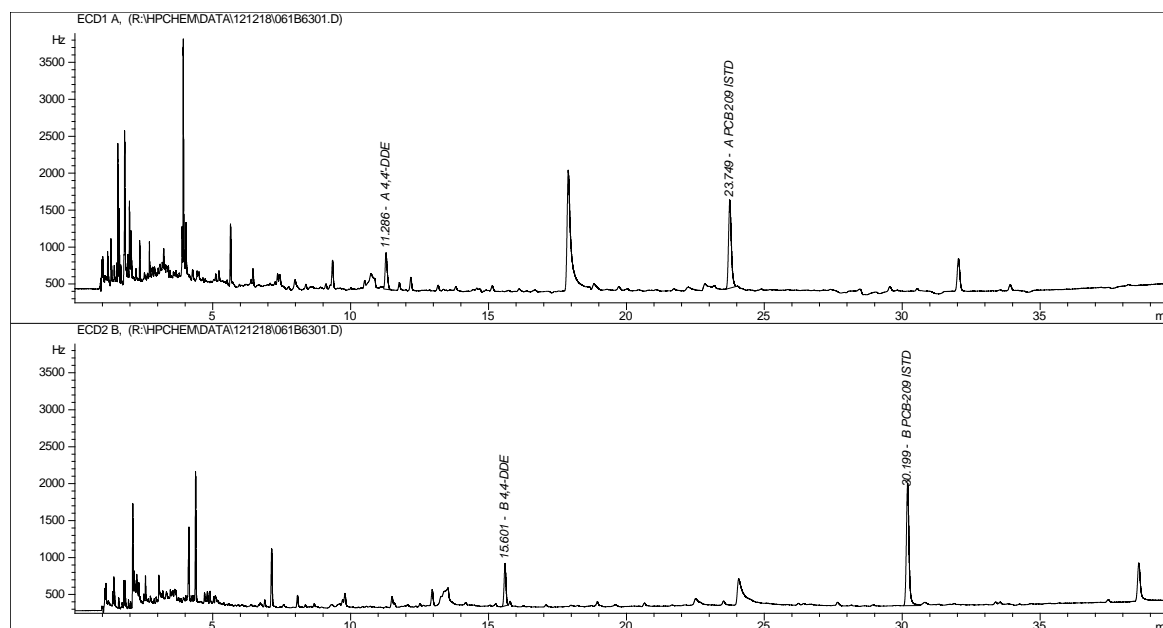


Figure 1. A typical GC/ECD dual channel chromatogram of a breast milk sample (HE) using cooled centrifugation sample processing method. Conditions: Column A: Stx-CLPesticides from the Restek (30 m x 0.25 mm x 0.20 μ m, Cat No. 11543) and Column B: Stx-CLPesticides2 from the Restek (30 m x 0.25 mm x 0.20 μ m, Cat. No. 11443) temperature program, 90°C (1.6 min), 50°C/min 170°C, 3.5 °C/min 300°C; injection mode, splittles 1 μ l (1 min) at 270°C; carrier H_2 (13.01 psi); detector ECDs at 330°C with N_2 make-up gas.

The DDE and PCB 209 peaks were in disturbance free region of both chromatograms. The calibration curve shows excellent 0.99908 correlations in 0.2-50 μ g/l concentration range of DDE according to Figure 2. The channel B shows 0.99725 correlation in the same range.

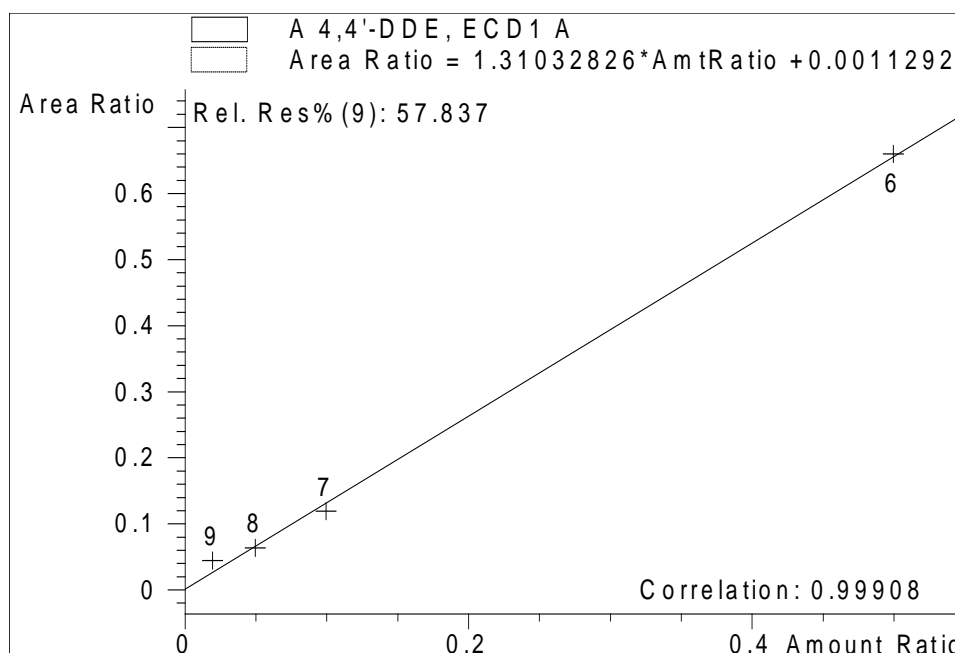


Figure 2 Calibration curve of DDE between 0.2-50 µg/l concentration ranges using 100 µg/l PCB 209. Column is Stx-CLPesticides2 from the Restek (30 m x 0.25 mm x 0.20 µm) at Chanel B.

DDT was not detectable in any any cases. The DDE was quantified in 6 samples of 7 tested milk samples (Table 1.). One of the mothers (VS) is a vegetarian, whose breast milk had not contained measurable DDE. Her data were omitted from further study. These results show the absent of recent DDT pollutions. Namely the high ratio between of DDE and DDT (DDT/DDE) show old pollutions (Mikes, 2012; Lotz, 2007). It is interesting to note, that every women was born after the DDT was banned in Hungary. These examples make obvious that the pollutions of DDT and its metabolites do not disappear during one cycle, but they pollute several cycles along the food chain.

The mean value is 102 µg/kg DDE in milk fat (2.71 µg/l milk) in the tested breast milk samples using cooled centrifugation sample treatment methods. This value is significantly lower than was measured in WHO surveys (2006, 2002 and 1997) in Hungary. The data of Table I. are not enough for exact statistical evaluation, however, some tendency can be observed.

The extraction procedures are appropriate, because the found 6.07 µg/kg result show 117% recovery for reference sample (spiked at 5 ppb level). The reproducibility seems good, because the sub-samples of the same mother (JKI, JKII) gave rather same results 2.43 µg/l and 2.41 µg/l concentration of DDE.

The QuEChERS method is appropriate to establish the DDE content of breast milk. The cooled centrifugation gave 4.72, and QuEChERS resulted in 4.04 µg/l DDE content in the case of HE mother. The MJ mother showed 1.66 µg/l with cooled centrifugation and 2.12 µg/l with QuChERS method for her breast milk. There are loose correlation ($r^2 = 0.7995$) between the fat content of breast milk samples and their DDE content. The ages of mothers and DDE content of milk fat show also a weak correlation ($r^2: 0.4425$). This effect can explain the bioaccumulation feature of then DDE. No correlation ($r^2: 0.0169$) was observed between the weight of the mother and DDE content of their milk. Probably, the weights of mothers do not represent alone the weight of their fat tissues.

Conclusion

The breast milk of Hungarian mothers contains DDE even today. No recent DDT pollution was recognized. The DDT and its metabolites are not disappear after one cycle, but they reaming during several cycles. This establishment is supported that the tested mothers were born after the ban of DDT in Hungary. The level of DDT level decreases slowly in breast milk. The eating habit may influence the DDE content of breast milk. The global warming has a possible risk for spread of malaria, therefore the DDT analyses remain active task for environmental protection.

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Table 1. DDE contents of the breast milk of the tested mothers

Sample I.D.	Age of mother (year)	Weight of mother (kg)***	Milk volume (ml)	Fat content in milk (g)	DDE concentration (µg/l) in	
					milk fat	milk
Reference*	-	-	25	0.627	169.8	4.26
VG	29	73	25	0.944	41.6	1.57
MJ	30	72	50	1.86	83.15	1.66
MJ/Q**	30	72	10	-	-	2.12
JK/I	32	62	25	0.872	69.0	2.41
JK/II	32	62	25	0.893	68.2	2.43
FV	33	70	25	0.627	43.0	1.08
HE	34	72	50	3.9	236.0	4.72
HE/Q**	34	72	10	-	-	4.04
VS	35	62	25	0.885	< 20	< 0.5
KD	36	61	25	1.004	90.5	4.26

*spiked cow milk

**analyzed with QuEChERS method

***weight of the mothers before their pregnancy

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