Determination of Recent Concentration of DDT and its Metabolites in Breast Milk in the Teaching of Behaviors of Persistence Organic Compounds

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Abstract: The problem of the persistent organic pollutants (POP) is an important part of the education program of Environmental Studies. The literature data refer several unwanted side effects of the intensive use of DDT. On the other hand the widely spread malaria epidemic infection makes the use of DDT necessary even today. The occurrence of DDT and its metabolites is a good representative of the persistency of such pollutants in the human breast milk. The samples were donated by university students and young teachers. This way the students could get personal involvement of the topic and measurements. The conventional and QuEChERS sample treatments were applied and their effectiveness were compared. GC-ECD instrumental analyses were used with parallel columns to demonstrate the selectivity and sensitivity of the required analysis. The results demonstrate the presence of DDE traces in breast milk of the majority of Hungarian mothers, even after the agricultural use of DDT was banned in 1968. No fresh DDT pollution was recognized in the tested samples. Correlations were established among the DDE contents of the breast milk samples and the ages and weights of the tested mothers. The measurements also show the persistency and biomagnificational feature of DDT. The educational aspects of each step of the applied procedures are emphasized in this paper. The analytical processes allow a deep insight for the environmental analysis for environmental engineering students. These offer a manual and theoretical practice with real samples, for improving the environmental attitudes.

Keywords: DDT and its metabolites; human breast milk; education of features of POPs

1 Introduction

The DDT (dichlorodiphenyltrichloroethane) was synthetized in 1874, and it was applied as pesticide since 1939. The worldwide production amount of DDT exceeded the 2.6 million tons, from 1950 to 1993, which is the largest quantity among the pesticides [1]. In Hungary, approximately 40 000 tons of DDT were used between the 1945-1966 period [2]. The 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 [3]. It is worthy to note that Hungary was the first country, which withdrew DDT from agricultural use in 1966 [2]. On the other hand the DDT remain in use as indoor DDT spraying against the malaria epidemic cases, because it is the most effective material against malaria transmitting insects (malaria mosquito anopheles) [4,5]. The analysis of DDT and other POP compounds are an important part of the knowledge of environmental engineering B.Sc. students.

The chemical structure of DDT and its main metabolite are in (Fig. 1) [6].

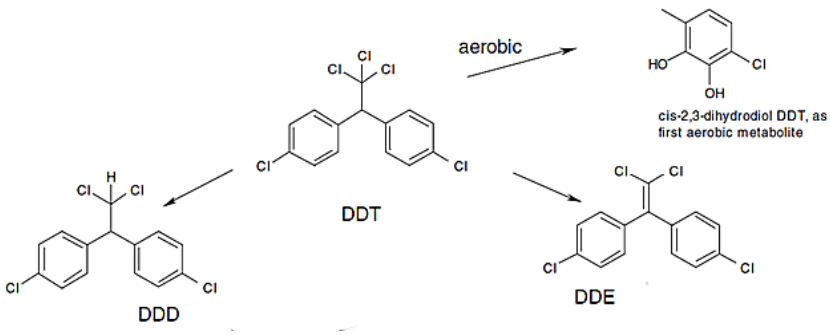

Figure 1
Structure of DDT (1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane) and its main metabolite DDD and DDE [6]

The DDT has low volatility (vapor pressure 2.53×10^{-5} Pa at 20°C) and lipophilic character (practically insoluble in water). These data suggest that the DDT and metabolites reach the human body through the food chain, with the exception of the direct inhalation of sprayed aerosols as mentioned in the literature in several times [2, 7]. DDT has very high persistency with 3-15 years half-life in soil, and DDE has even much stronger persistency than DDT [8, 9]. The high DDE/DDT ratio show old pollution, but low DDE/DDT ratio is evidence of recent DDT pollution [9]. The enantiomer ratios of *o,p*-DDE also provide information of origin of pollution [10].

The DDT is very effective insecticide, having neurotoxic (sodium channel opening) effect in insects [11]. The DDT had a very high popularity because it seemed to be a very effective pesticide and harmless for warm blooded animals.

Later several pests became resistant against DDT, and serious unwanted side effects emerged for fishes and warm blooded animals [11, 12].The most shocking observations were written for the public in the "Silent Spring", the book of Rachel Carson [12]. The facts of this book help to improve the environmental awareness. Topics of the book are partly valid even recently, therefore is an important literature for students.

The DDT has tumorigenic, neurotoxic, estrogenic endocrine disruptive effects for the warm blooded animals and humans [11, 12, 13, 14, 15]. One of the most dramatic symptoms was the egg thinning of predatory birds, which was the reason for drastic decline of predatory birds in several areas [13, 14]. The estrogenic effects of DDT caused a drastic shift of sex ratio of gulls toward the female [15], therefore often two females nested together. Not only the DDT, but their main metabolites show the toxic effects. DDE proved more toxic than DDT in several cases. This example demonstrates well, not only the mother compounds, but their metabolites have toxic effects in the environment. Important characteristic of the POP load of the body is the TDI (tolerable daily intake) value for the human intake of pesticides, which is at Σ DDT 10 μg/body kg [2].

Another unwanted feature of DDT and its metabolites are their bioaccumulation features [7]. Bioaccumulation meaning, the lipophilic DDT and its metabolites depositing in adipose tissues and they can hardly be mobilized. On the other hand, they can easily be mobilized by expression of breast milk, because the milking metabolism being different from normal excretion [7, 8, 16, 17]. Students can learn that the toxic compounds can become enriched in different tissues and mobilize in different ways .The biomagification accelerates the issue, because the levels of pollutions multiply even several million times along the food chain [7, 13]. The long range transport and biomagnification of DDT and its metabolites cause high level pollution in living creatures on the top of food web (human and polar bears) in the Arctic Area, where the DDT was never applied [14]. These facts demonstrate, how pollution can appear in unexpected places even in high concentrations. Several publications dealt with the increased amounts of DDT in human breast milk. The DDT levels increase with the age of mothers [11, 17, 18], body mass index [11, 21] fat reach eating habits [7, 8, 18]. The concentration levels of DDT and metabolites are higher in the milk of primiparae mothers in cases of high pollution levels [7, 19, 20].

On the other hand, no significant differences were found between DDT levels of breast milk of primiparae and multiparae mothers, in the cases of the low pollution levels [8, 16, 17]. Recently, the lactated babies get several times lower amount of DDT and its metabolites than the TDI in Europe [17], but the pollution level of them exceed TDI in those territories, where DDT is used against malaria [16, 21].

These facts show obviously to the students that a compound with very useful effects can cause several unwanted side effects too. 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 withdrew DDT from agricultural use in 1966.

The highly contaminated areas of malaria, however, allow the recent use the DDT against malaria mosquito, mostly as indoor spraying [3, 4, 5, 9, 16]. This limited use of DDT seems to be a good compromise. The global warming results in an estimated threat of malaria cases [22], which keeps the DDT monitoring active, even in Europe.

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 [7, 8, 16, 17]. The situation has become worse, because the newborn infants are in rather sensitive period of their life for endocrine disruptive compounds. These are the reasons why national and international campaigns were launched for the tests of DDT and its metabolites content of mother milk [20]. The well-established analysis methodology of DDT also helped the success of this campaign.

The low concentration levels of DDT and metabolites and the difficult matrix require multistep sample pretreatment and dedicated instrumental analysis [23, 24]. The WHO protocol does not favor any sample pretreatment and instrumental method, it allows for use of well-trained method of the analyzing laboratory [20]. Such examples help to build up an environmental approach, because the students can be personally involved.

Several methods were developed, but we choose the efficient SPE methods, because they are selective and they are good to concentrate the trace components. Recently the universal QuEChERS (Quick, Easy, Cheap, Effective, Rugged, Safe) methods have become more and more popular [25, 26]. These multiresidue methods are rather fast, easily managed, and require low solvent consumptions. These methods are based on acetonitrile extraction, and centrifugation followed by dispersive solid-phase extraction. The method was invented for analysis of vegetables and fruits. The modified QuEChERS method, however, can be applied for samples with high lipid content too [27, 28]. In these cases additional fat removing steps are advised. On the other hand, the QuEChERS method has not yet been applied frequently for breast milk samples. We decided to compare the QuEChERS method with the traditional cleaning method. An important task of higher education is to teach the recent best available techniques (BAT), such as the QuEChERS method.

GC/ECD or GC/MS methods are appropriate for instrumental analysis of DDT and its metabolite content of breast milk samples [23, 24, 26]. Dual - column analysis methods confirm the compounds' identification in the samples. The

parallel joint columns produce two retention times, and the same peak areas for one compound, which make the sample identification certain. This dedicated instrumentation shows the requirements of trace analysis.

Our aims with this report were the following:

- To show the recent presence of DDT and its metabolites in breast milk samples in Hungary, for the environmental engineering BSc students. This project is also good to involve the students in the scientific research of our institute.
- To see the tendency of concentration of the DDT and its metabolites in time scale and other parameters. In this way the student can learn the main tendencies of the POP pollutions.
- To prove the applicability of the QuEChERS method for breast milk samples showing a method of future.
- To give ability of manual work for students, in this way they can get experiences, which may be of use in their future jobs.
- To show that DDT is not an outdated analysis task even in Hungary. The possible threat of malaria keeps DDT analyses alive, even in Europe. Moreover, the bleed of illegal hidden depots can cause recent DDT pollutions too.

2 Experimental

2.1 Chemicals

Residue grade solvents (acetonitrile, dichloromethane, hexane, acetone) and anhydrous Na_2SO_4 were purchased from Sigma Aldrich. The used QuEChERS extraction kits (P/ 5982-5650) containing salt mixtures (4 g $MgSO_4$; 1 g NaCl; 1 g NaCitrate; 0.5 g disodium citrate sesquihydrate) The QuEChERS dispersive kit for SPE (P/N 5982-0028 2, Agilent) includes Eppendorf tube filled with 50 mg PSA, 50 mg C_{18}, 7.5 mg GCB, 150 mg $MgSO_4$. Following standards were applied: Organochlorine Pesticide mixture from the Restek (Cat. no. 32415), and PCB 209 from the Riedel-de Häen (Cat No. 35587).

2.2 Instrumentation

The cooled (-10 $^{\circ}$C) and room temperature centrifugations were done in a laboratory centrifuge (Hermle) at 3000 rpm.

An Agilent Technologies 6890N Network GC System with HP7683 autosampler was used for dual μGC/ECD analysis. Column pair was mounted in a press-fit Y-shaped glass 3-way union splitter. Parallel columns arrangement was used to overcome the coelution and identification difficulties. Two Restek produced columns were the following:

- Column A is Stx-CLPesticides (30 m x 0.25 mm x 0.20 μm, Cat No. 11543).
- Column B is Stx-CLPesticides2 (30 m x 0.25 mm x 0.20 μm, Cat. No. 11443). Parallel columns arrangement was used to overcome the coelution and identification difficulties.

2.3 Procedures

2.3.1 Collection of Breast Milk Samples (Sampling and Sample Preparation of Breast Milk Samples

The tested persons were students and young teachers. In this way the students have personal contact with samples, because they know the donors. 8 breast milk samples were collected. The ages of mothers varied between 20-32 years. The mothers had their first delivery in seven cases, because it is expected that the DDT and its metabolite have the highest content in the case of first delivery [7, 20]. The samples were collected in the 3-10 weeks after they gave birth. The milk samples were stored in deep freezer until the extractions. The students came to know the basic rules of sampling and sample storages.

The frozen samples were let to thaw and then the liquids were homogenized. Method 1 required 25-50 g of milk portions using the EN 1528 method [23]. Method 2 used 10 g of samples for the QuEChERS method [26]. 1 μg PCB 209 surrogate standard solutions were added to every sample. The use of surrogate standards show, how one can eliminate the effect of sample loss during the sample treatments.

The reference sample was 25 ml commercialized cow milk which was spiked at 10 μg/kg level with the organochlorine pesticide mixture containing the DDT and its metabolites. This step drew the attention of students to the necessity of reference samples to eliminate the matrix effects.

2.3.2 Method 1: Sample Processing using Cooled Centrifugation (EN 1528-2:1998 (Point 6.1.4 Extraction by Cooled Centrifugation) [23]

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 Na_2SO_4. The hexane was evaporated and the weights of the remaining fats were measured.

0.5 grams of fats were molten on hot water baths, and mixed with 3 ml extraction solutions (ACN/CH_2Cl_2- 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 N_2 and the final volumes were adjusted with hexane to 1 ml. These solutions were taken into GC vials and analyzed by GC-dual ECD.

2.3.3 Method 2: Sample Processing with the QuEChERS Method (EN 15662:2009) [26]

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 by centrifugation (4000-4500 rpm) for 15 min. The solutions were decanted into test tubes and kept in deep freezer overnight in order to remove the major part of the 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 C_{18}, 7.5 mg GCB, 150 mg $MgSO_4$). 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 analyzed by GC-dual ECD.

2.4 Analysis with GC-ECD Instrument

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 splittles 1 µl (1 min) at 270 °C. H_2 was the carrier gas with 13.01 psi inlet pressure. Detectors were µECDs at 330 °C using N_2 make –up gas.

The chromatograms were treated with ChemStation software (Agilent). Calibration curves are built/drawn in 0.2 -50 µg/l concentration range of DDE.

3 Results and Discussions

The chromatograms were appropriate to determine the compound of interests (Figure 2).

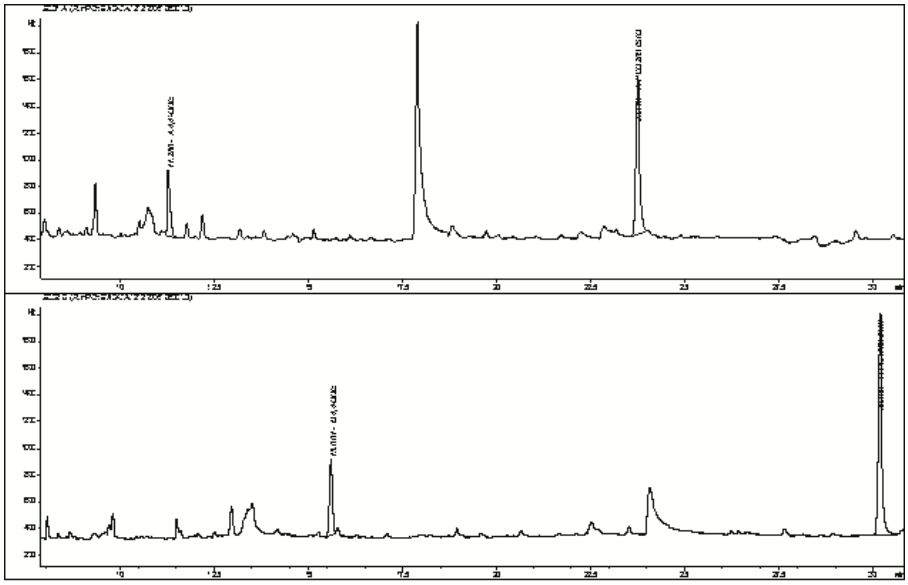

Figure 2

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 µm, Cat No. 11543) and Column B: Stx-CLPesticides2 from the Restek (30 m x 0.25 mm x 0.20 µm, 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 µl (1 min) at 270 °C; carrier H_2 (13.01 psi); detector ECDs at 330 °C with N_2 make–up gas. Retention times are 11.286 min for 4,4'-DDE and 23.749 min for PCB 209 ISTD on Column A, and 15.601 min for 4,4'-DDE and 30.199 min for PCB 209 ISTD

The DDT, DDE and PCB 209 peaks were in disturbance free region of both chromatograms as results of effective sample cleaning and the use of very pure chemicals. The attention of students were drawn the importance of residue grade solvent, which is important for trace analysis, which becomes more emphasized when electron capture detector is used.

DDT was not detectable in any cases. The high ratio between DDE and DDT (DDT/DDE) shows old pollutions [2, 8]. It is interesting to note, that every mother was born after DDT had been banned in Hungary. Based on these examples, it is obvious, that the pollutions of DDT and its metabolites do not disappear during one cycle, but pollute several cycles along the food chain. The remaining DDE is a very demonstrative example for the persistency of chlorinated hydrocarbon pesticides. The presence of DDE without any detectable amount of DDT highlights the fact, that it is not enough to look for the compound applied, but the analysis of its metabolites is also important.

Therefore the calibration curve was constructed only for DDE. The calibration curve shows excellent 0.99908 correlations in 0.2 -50 µg/l concentration range of

DDE according to Figure 3. The channel B shows 0.99725 correlations in the same range.

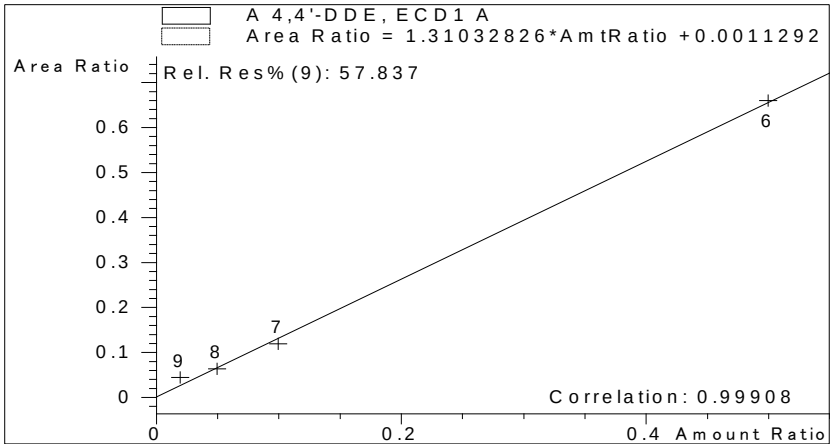

Figure 3

Calibration curve of DDE between 0.2-50 µg/l concentration range 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.

DDE was quantified in 9 samples of 10 tested milk samples (Table 1). The milk sample of one mother (JK) was divided two part to check the reproducibility of method One of the mothers (VS) is a vegetarian, whose breast milk had not contained measurable DDE. Her data were omitted from further study. On the other hand the milk sample of VS was a good example, for the correlation between the eating habits of mothers and POP contaminants of their milk.

The mean values are 102 µg/kg DDE in milk fat and 2.71 µg/l 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 1997) in Hungary.

The data of Table 1 are not enough for exact statistical evaluation; however, the students may observe the typical tendencies.

The extraction procedures are satisfactory, and 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 one mother (JKI, JKII) gave rather same results 2.43 µg/l and 2.41 µg/l concentration of DDE.

The modified QuEChERS method is also appropriate to establish the DDE content of breast milk (Fig. 4). The QuEChERS method is a universal sample pretreatment method, therefore its recovery can smaller for certain compounds than the special sample pretreatments methods for their targets compounds. This effect is well illustrated by the results of MJ and MJQ samples.

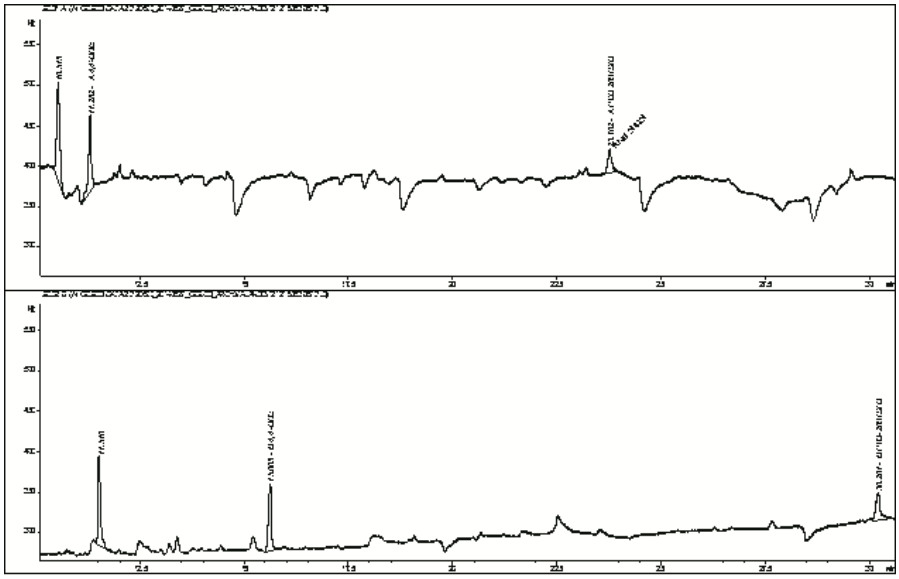

Figure 4

GC/ECD dual channel chromatogram of a breast milk sample (MEQ) using QuEChERS sample processing method. Conditions: Column A: Stx-CLPesticides from the Restek (30 m x 0.25 mm x 0.20 µm, Cat No. 11543) and Column B: Stx-CLPesticides2 from the Restek (30 m x 0.25 mm x 0.20 µm, 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 µl (1 min) at 270 °C; carrier H_2 (13.01 psi); detector ECDs at 330 °C with N_2 make–up gas. Retention times are 11.282 min for 4,4'-DDE and 23.762 min for PCB 209 ISTD on Column A, and 15.605 min for 4,4'-DDE and 30.207 min for PCB 209 ISTD.

The cooled centrifugation gave 4.72 µg/l, 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 QuEChERS method for her breast milk.

Table 1

DDE contents of the breast milk of the tested mothers

Sample I.D.	Age of mother	Weight of mother***	Volume of the milk	Fat content of the milk	DDE conc. of the milk fat	DDE conc. of milk
Unit	year	kg	ml	G	µg/l	µg/l
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

There are loose correlations (r^2= 0.7995) between the fat content of breast milk samples and their DDE content.

The HE mother had the highest fat content in her milk (7.8%) and her milk showed highest (4.72 μg/L) DDE content from the non-vegetarian mothers. The milk sample of FV produced the lowest fat content (0.627%) and her milk contained lowest, only 1.08 μg/L DDE content. This data shows that the eating habit influenced the DDE content of milk sample. Lipophilic character of DDE could also conclude from correlation of the fat content of breast milk and their DDE content.

No correlation (r^2: 0.0169) was observed between the weight of mothers and DDE content of their milk. Perhaps their body mass index may correlate to the DDE contents of their milk.

The ages of mothers and DDE content of milk fat also show a weak correlation (r^2: 0.4425). This effect can explain the bioaccumulation feature of then DDE. The KD sample, a mother aged 36 had second highest DDE content (4.26 μg/L) in her milk. The VG, the youngest at 29, had second lowest DDE content (1.57 μg/L) in her milk.

The data of Table 1 also presents, that the samples from biological origin could produce a big deviation (s: 1.31). The highest DDE content was almost four times higher than the lowest content.

Conclusion

These experiments and results of this paper served as an excellent tool for the education of POP in the environmental engineering courses. The relatively low sample numbers do not allow for exact statistical evaluation, but the main tendency has been manifested. No recent DDT pollution was recognized in the breast milk samples. On the other hand, the overwhelming part of the breast milk samples of Hungarian mothers contain DDE even today, showing the high persistency feature of DDT and its metabolites. All of the samples contained less DDE than tolerable daily intake. The DDT and its metabolites have not disappeared after one cycle from the food chain, but they remain for several cycles. This establishment was supported by the fact that the tested mothers were born after the ban of DDT in Hungary. Eating habits may influence the DDE content of breast milk, because all of the samples contained DDE except the milk

of a vegetarian woman. The used sample preparation methods were appropriate to establish the DDE contents of the sample. The results of modified QuEChERS method are comparable with the results of traditional cold centrifugation method. Loose correlations were found between the age, fat content of milk samples and their DDE content.

These results demonstrate the requirements of developed instrumental background and laborious sample treatments for environmental trace analysis. The established correlations show the features of POP in biological matrices. The analysis of DDT and its metabolite cannot be omitted from the education of environmental engineers; in spite of agrochemical use of DDT was having been banned for decades. Since global warming carries a possible risk for spreading of malaria, DDT analyses remain active task for environmental protection.

The established results demonstrate the following:

- The analysis of DDT and its metabolite is good example for the persistency bioaccumulation and biomagnifications of POP compounds.
- The measurements thought the appropriate sampling, sample storage, sample pretreatments, and instrumental measurements.
- These tests brought personal involvements for the students to build up their environmental attitudes.

Acknowledgment

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