Development of analytical method for determination of organochlorine pesticides residues in meat by GC-ECD

RUMYANA T. DIMITROVA1, ISKRA I. STOYKOVA1, TÓDORKA T. YANKOVSKA-STEFANOVA2, SPASKA A. YANEVA3, TODOR T. STOYANCHEV**

1Central Laboratory of Veterinary Control and Ecology, Bulgarian Food Safety Agency, Sofia, Bulgaria  
2Bulgarian Food Safety Agency, Sofia, Bulgaria  
3University of Chemical Technology and Metallurgy, Sofia, Bulgaria  
*Corresponding author: todor.stoyanchev@uni-sz.bg

SUMMARY

Aiming to meet the European requirements for monitoring of pesticide residues in food of animal origin, the analytical scope of the methods in Bulgaria need to be extended. Herein, we introduce a new method that performs better analytical characteristics and allows more compounds to be determined, compared to the existing method used for determination of organochlorine pesticides in meat. So far, in the national monitoring program a total of eight organochlorine pesticides have been analyzed: aldrin, heptachlorepoxyde– trans isomer, HCH – α, β, γ isomers, p,p’-DDE, p,p’-DDD and p,p’-DDT, by means of a method based on EN 1528:2001 – Fatty food – determination of pesticides and polychlorinated biphenyls (PCBs). Using our new method, it is possible seven additional pesticides to be determined: dieldrin, endosulfan-α and β isomers, endosulfan sulfate, HCB, heptachlor, heptachllorepoxide– cis isomer. The method is based on liquid-liquid partitioning with acetonitrile– n-hexane, clean-up step on a home-made silica gel column and GC-ECD determination of pesticides. The validation is performed according to the recommendations in the document SANTE/11945/2015 and it meets the acceptability criteria. The proposed method was successfully used in laboratory proficiency test where OCPs included were HCH isomers - β (Z-score of rezult: 0.1), p,p’-DDE (Z-score of rezult: -0.6), Endosulfansulfate (Z-score of rezult: 0.5).

Keywords: organochlorine pesticides in meat, sample preparation, GC-ECD determination, clean-up method, liquid-liquid partitioning

RÉSUMÉ

Développement d’une méthode analytique pour la détermination des résidus de pesticides organochlorés dans la viande par GC-ECD

En vue de satisfaire aux exigences européennes en matière de surveillance des résidus de pesticides dans les denrées alimentaires d’origine animale, le champ d’analyse des méthodes en Bulgarie doit être étendu. Nous introduisons ici une nouvelle méthode qui permet d’obtenir de meilleures caractéristiques analytiques et permet de déterminer plus de composés par rapport à la méthode actuelle utilisée pour la détermination des pesticides organochlorés dans la viande. Jusqu’à présent, dans le cadre du programme de surveillance national, huit pesticides organochlorés ont été analysés: Aldrin, Heptachlorepoxyde – isoème trans, isoèmes HCH - α, β, γ, p,p’-DDE, p,p’-DDD et p,p’-DDT, au moyen d’une méthode basée sur la norme EN 1528 : 2001 - Aliments gras - Détermination des pesticides et des polychlorobiphényles (PCB). En utilisant notre nouvelle méthode, il est possible de déterminer sept autres pesticides: dieldrine, endosulfan α et β isoèmes, endosulfan sulfate, HCB, heptachlor, heptachlorepoxyde-cis isoème. La méthode est basée sur la technique de l’extraction par solvant accélérée, le cloisonnement liquide liquide avec de l’acétonitrile - n-hexane, le nettoyage sur colonne de gel de silice et la détermination des pesticides par GC - ECD. La validation est effectuée conformément aux recommandations du document SANTE / 11945/2015 et répond aux critères d’acceptabilité. La méthode proposée a été utilisée avec succès dans un test de compétence en laboratoire où les OCP inclus étaient des isoèmes HCH – β (score Z de rezult: 0.1), p,p’-DDE (Z-score de rezult: -0.6), Endosulfansulfate (Z-score de rezult: 0.5).

Mots clés : Pesticides organochlorés dans la viande, préparation des échantillons, détermination de la GC-ECD, méthode de nettoyage, cloisonnement

Introduction

Among the persistent organic pollutants (POPs), organochlorine pesticides (OCPs) are the most hazardous class, which have been banned for agricultural and domestic uses in Europe according to the Stockholm convention, due to their environmental persistence and potential adverse effect on wildlife, animal and human health [6]. As a consequence of their bio-accumulation and long half live, their residues can further be found in food products of animal origin as meat, fish, eggs, milk, etc, especially in tissues and foods with high fat content [17]. European legislations establish maximum residue levels (MRLs) for pesticides in or on food and feed of plant and animal origin in Regulation (EC) 396/2005 [22]. All European Union member countries must collect and analyse samples for the needs of national monitoring plan according to Council Directive 96/23/EC, which set measures to monitor substances and residues in live animals and animal products [11]. Concentrations of organochlorine pesticides in animals and meat should be decreasing during the years which will proof that they are not in a use in the current agriculture. Stringent coordinated multiannual control program as a measure for minimize exposure of population to pesticide residues in food is been established with Regulation (EU) 662/2016, where mandatory monitored pesticides (including organochlorine pesticides) are presented [8]. Thus in order to meet the current legislative requirements as well to carry out the
European monitoring programs, for the food laboratories it is necessary the development of routine, sensitive and specific analytical methods for determination of organochlorine pesticides in food. Sample preparation is still the major challenge in the analytical methods for food residues. For the routine laboratories of food control it is important to have robust, simple and rapid extraction procedure suitable for multiresidue application. There the main point is extending the analytical scope of the methods used for multiresidue analysis in one instrumental assay, but with specificity that some of the newly added OCPs have very low MRLs, and others cannot be extracted from the lipid fraction via florisil column, that is common used so far.

OCPs are lipophilic compounds and they are extracted from the food matrix with fat extracting techniques. Traditionally, the preliminary sample preparation of complex samples, such as meat and meat products, is carried out in a sequence of extraction and clean-up procedures. For this reason, sample extraction can be long and tedious, involving several clean-up steps to remove the fat and other co-extracts. In his review Martial LeDoux [17] summarized that the most widely used pesticide extraction technique from foods of animal origin are: solid–liquid extraction, the traditional Soxhlet extraction method, accelerated solvent extraction (ASE), microwave-assisted extraction, matrix solid-phase dispersion. After extraction the clean-up and enrichment steps are mainly performed by: freezing centrifugation, liquid–liquid partitioning, gel permeation chromatography (GPC), solid-phase extraction, etc. [17].

Most of the procedures are unpleasant for routine analysis and fail as complicated, time consuming, tedious or lack in multipesticide application. Nowadays, QuEChERS method (quick, easy, cheap, effective, rugged and safe) is widely used as extraction and clean up method for multiresidue pesticide analysis and multiple food samples and many modifications appear covering changes in extraction solutions, buffer acidity etc. [1, 2, 9, 18, 19]. All steps during the sample preparation aid the instrumental analyses the cleaner the extract resulted in the lower determined residue concentration by the detector [27]. Disadvantage of the multistep procedures is a risk for analyte losses and uses of large amount of expensive and hazardous organic solvents. A newel trend in pesticide analysis is environmental friendly approach which should be easy to apply and use lower amount of organic solvents and low cost materials.

The aim of this study is focused on the development, optimization and validation of a simple and cheap method for sample preparation, extraction, clean-up and multiresidue determination of OCPs in meat and foods of animal origin using conventional GC–ECD. The main point is increasing the number of analyzed OCPs in one multiresidue assay and reaching the limit of quantification at least twice lower than MRLs. The challenge is precisely removing the lipid and coextracts content from the pesticide extract.

**Materials and Methods**

**SOLVENTS AND REAGENTS**

Hexane, cyclohexane, acetonitrile, tolune (gradient grade) and anhydrous sodium sulphate were supplied by Supelco (USA). Nitrogen (99,999% purity) was used as carrier and make up gas.

**ANALYTICAL STANDARDS**

For the method evaluation were used organochlorine pesticide standards (purity > 96%; Sigma Aldrich, Germany) including individual: aldrin, hexachlorocyclohexane (HCH) isomers - α, β and γ (lindane), heptachloropoxide (HCE) cis and trans isomers, p,p'- DDT, p,p'- DDE, p,p'- DDD, hexachlorobenzene (HCB), endosulfan-α and β isomers, endosulfan sulfate, heptachlor, dieldrin, Mirex were used as internal standard also in the method evaluation. Primary stock solutions of each pesticide analyte were prepared in hexane at a concentration of 1000 μg/mL. Additional single analyte solutions were prepared in hexane at a concentration of 1 μg/mL. Multi-pesticide solution was also prepared in hexane with final concentration 0.01 μg/mL for γ-HCH and 0.05 μg/mL for all the other pesticides. Internal standard solution of Mirex was used in concentration of 1 μg/mL.

**EQUIPMENT**

Chromatographic analyses were achieved by gas chromatograph system coupled with electron capture detector (ECD) (Trace-GC, Thermo, USA). Separation of pesticides was carried out with a capillary column DB-17MS (30 m, 0.25 mm internal diameter, 0.25 μm film thickness)
ANALYTICAL METHOD

Sample preparation

Lipophilic pesticides and fat extraction and determination of the total fat content in the sample:

Organic solvent fat extraction technique was used together for total fat content and organochlorine pesticide residues assay, according to EN 1528 [3]. Total fat content in the meat sample was determined gravimetrically.

Blank meat sample (ca 250 g) was homogenized in a powerful blender. From the homogenized sample 10 g were mixed in a mortar with 5 g of anhydrous sodium sulfate for 1 minute and then transferred into extraction cell of accelerated solvent extraction machine. The total fat extraction was performed for 20 min. at 100°C in 1500 psi pressure by using 135 ml solvent per cell. Mix of toluene and cyclohexane in ratio 1:1 (v/v) was used as a solvent. After 20 min of extraction, the liquid part from the Dionex cell was transferred into a dry glass flask. Each empty flask was weighted individually with accuracy of 0.0001 g. Solvent was rotary-evaporated until constant weight and the flask was weighted again. The total fat content in the meat sample was calculated by the equation:

\[ \text{%Fat} = \frac{W_1 - W_2}{W_1} \times 100 \]

- \( W_1 \) = Sample weight (10 g)
- \( W_2 \) = Empty flask weight
- \( W_3 \) = Flask weight after evaporation

Liquid-liquid partitioning

Liquid-liquid partitioning was carried out with hexane and acetonitrile and it was used to remove the main portion of fat content.

After rotary evaporation, 0.5 g of extracted fat was transferred into a 50 ml polypropylene centrifuge tube and 50 µl from the stock solution of Mirex (internal standard) were added. After few min. 5ml of hexane was put in and well homogenized by vortexing. Pure acetonitrile 5 ml was transferred to the mixture and vortexed again for about 2 min, centrifuged for 2min at speed equivalent on 358 g (2000 rpm) for better separation of the two liquid layers (hexane and acetonitrile). Only the upper hexane layer was collected to another polypropylene centrifuge tube and the extraction with acetonitrile was done in duplicate. The remaining liquid layer was partitioned again with acetonitrile in duplicate. All acetonitrile portions were mixed together in to glass tube and placed at 50°C water bath and total extract evaporated to dryness under nitrogen steam.

Additional purification step on mini silica column

In our method the additional purification of lipids and other coextracts was performed on a homemade mini-silica column. The column was packed as follows (from bottom to top): cotton wool, 1.5 g of silica gel and about 5 mm of anhydrous sodium sulfate. Immediately before use, the column was conditioned with 2 ml hexane. The dry extract from liquid-liquid partitioning was dissolved in 2 ml of toluene, homogenized by vortexing and all the volume flashed by pipetting throughout the mini-silica column. The column was next eluted with 6 ml of toluene and all the eluent was collected in glass tube and evaporated to dryness under nitrogen steam. The dry extract was dissolved with 0.5 ml cyclohexane and transferred to a 2 ml glass-vial for GC determination.

Chromatographic conditions:

Analysis of pesticides residues was carried out with a gas chromatograph system coupled with ECD. The ECD temperature was set at 300°C. Nitrogen was used as a carrier gas with a flow rate of 1.1 ml/min. The injection volume was 2 µl with a splitless injection mode. Injector S/SL was heated on 250°C with the following injection program for oven: 80°C (hold 1 min), 15°C/min to 120°C, 5°C/min to 198°C (hold 5 min), 5°C/min to 280°C (hold 20 min). Pesticides were identified by comparing their retention time on chromatogram with those of the standard mixtures of OCPs by a GC software “ChromCard” (Thermo Fisher Scientific).

METHOD VALIDATION

The method validation was performed according to recommendations in the procedure of SANTE/11945/2015 [23]. For this purpose it was used meat with fat content more than 10 %, from which the fat was ASE extracted, and extracted fat used as a blank matrix where the results were expressed as µg/g fat according to Regulation 396/2005 [22]. The calibration curve was prepared, using blank material (0.5 g fat extracted from meat) spiked at five concentration levels corresponding to 0, 0.025, 0.05, 0.075, 0.1 µg/g for all analytes, except γ-HCH, where the levels correspond to: 0, 0.005, 0.01, 0.015, 0.02 µg/g by adding 0, 250, 500, 750, 1000 µl of the multi-standard solution and 50 µl of 1 µg/ml internal standard solution of Mirex at a level corresponding to 0.1 µg/g.

Validation procedure was carried out by blank material (0.5 g fat extracted from meat) spiked at three concentration levels corresponding to: 0.05, 0.075, 0.1 µg/g for all analytes, except γ-HCH, where the levels correspond to: 0.01, 0.015, 0.02 µg/g by adding 500, 750, 1000 µl of the multi-standard solution and 50 µl of 1 µg/ml internal standard solution of

---

Revue Méd. Vét., 2018, 169, 4-6, 77-86
Mirex at a level corresponding to 0.1 μg/g. (shown in Table I). Spiked blank at each concentration level were analysed in 3 series, each on the different day, and each in 6 replicates.

The method was evaluated by the obtained parameters for linearity, mean recovery, precision (repeatability and reproducibility), limit of quantification (LOQ) and uncertainty. Average recovery was calculated by comparing the determined concentrations of spiked samples to their target level. The LOQ is determined as the lowest concentration level that can be validated with acceptable values for recovery and precision. The reported uncertainty was calculated in accordance with the recommendations of EURACHEM / CITAC Guide CG 4, as expanded uncertainty with a coverage factor of 2 to give a level of confidence of approximately 95% [12].

<table>
<thead>
<tr>
<th>Pesticide</th>
<th>Linearity</th>
<th>Spike level [μg/gfat]</th>
<th>Recovery [%], (n=18)</th>
<th>Repeatability RSDr [%], (n=6)</th>
<th>Within-laboratory reproducibility RSDwR [%], (n=18)</th>
<th>Uncertainty [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCB</td>
<td>$y = 15.011x + 0.0194$ [R^2 = 0.9917]</td>
<td>0.05</td>
<td>55.5</td>
<td>10.8</td>
<td>16.7</td>
<td>14.6</td>
</tr>
<tr>
<td>α-HCH</td>
<td>$y = 15.219x + 0.0312$ [R^2 = 0.9863]</td>
<td>0.05</td>
<td>64.1</td>
<td>11.4</td>
<td>12.3</td>
<td>17.8</td>
</tr>
<tr>
<td>γ-HCH</td>
<td>$y = 19.859x - 0.0075$ [R^2 = 0.981]</td>
<td>0.01</td>
<td>63.1</td>
<td>8.4</td>
<td>16.4</td>
<td></td>
</tr>
<tr>
<td>β-HCH</td>
<td>$y = 21.72x + 0.0222$ [R^2 = 0.994]</td>
<td>0.05</td>
<td>89.9</td>
<td>5.5</td>
<td>11.3</td>
<td></td>
</tr>
<tr>
<td>Heptachlor</td>
<td>$y = 33.931x - 0.0348$ [R^2 = 0.9928]</td>
<td>0.05</td>
<td>56.3</td>
<td>4.8</td>
<td>10.4</td>
<td></td>
</tr>
<tr>
<td>Aldrin</td>
<td>$y = 41.48x + 0.046$ [R^2 = 0.9978]</td>
<td>0.05</td>
<td>70.3</td>
<td>6.5</td>
<td>14.3</td>
<td></td>
</tr>
<tr>
<td>HCE-cis</td>
<td>$y = 42.461x - 0.1029$ [R^2 = 0.9956]</td>
<td>0.05</td>
<td>69.8</td>
<td>9.4</td>
<td>8.1</td>
<td></td>
</tr>
<tr>
<td>HCE-trans</td>
<td>$y = 29.805x - 0.0095$ [R^2 = 0.9957]</td>
<td>0.05</td>
<td>65.2</td>
<td>4.9</td>
<td>14.5</td>
<td></td>
</tr>
<tr>
<td>α-Endosulfan</td>
<td>$y = 34.334x - 0.1095$ [R^2 = 0.9949]</td>
<td>0.05</td>
<td>79.1</td>
<td>4.8</td>
<td>8.3</td>
<td></td>
</tr>
<tr>
<td>p,p’-DDE</td>
<td>$y = 27.604x + 0.0061$ [R^2 = 0.9973]</td>
<td>0.05</td>
<td>68.4</td>
<td>2.1</td>
<td>10.6</td>
<td></td>
</tr>
<tr>
<td>Dieldrin</td>
<td>$y = 34.403x - 0.11$ [R^2 = 0.992]</td>
<td>0.05</td>
<td>66.0</td>
<td>9.6</td>
<td>9.5</td>
<td></td>
</tr>
<tr>
<td>p,p’-DDD</td>
<td>$y = 30.336x + 0.0601$ [R^2 = 0.9921]</td>
<td>0.05</td>
<td>75.8</td>
<td>3.7</td>
<td>14.1</td>
<td></td>
</tr>
<tr>
<td>β-Endosulfan</td>
<td>$y = 33.76x - 0.1408$ [R^2 = 0.9846]</td>
<td>0.05</td>
<td>60.8</td>
<td>4.9</td>
<td>8.0</td>
<td></td>
</tr>
<tr>
<td>p,p’-DDT</td>
<td>$y = 27.248x - 0.0085$ [R^2 = 0.9995]</td>
<td>0.05</td>
<td>73.2</td>
<td>6.7</td>
<td>6.9</td>
<td></td>
</tr>
<tr>
<td>Endosulfansulfate</td>
<td>$y = 19.901x - 0.024$ [R^2 = 0.9895]</td>
<td>0.05</td>
<td>81.9</td>
<td>6.2</td>
<td>12.6</td>
<td></td>
</tr>
</tbody>
</table>

Table I: Validation parameters of investigated 15 organochlorine pesticides compounds obtained in the method validation procedure by spiked blank material (fat extracted from meat).
Results

Fifteen organochlorine pesticides were chosen for evaluation of selectivity and specificity of the new method for qualitative determination by GC-ECD. Typical chromatograms obtained from the standard solution of OCPs, blank material, and spiked blank material showed clear separation of targeted 15 pesticides as a result of appropriate sample preparation, oven temperature programme and chromatographic conditions used (fig 1). All 15 OCPs were qualitative determined in spiked material at the lowest validation level. Developed sample preparation and analytical procedure in the used method resulted in separation each of the 15 OCPs analysed, without interference.
peaks between the pesticides or between matrix components and pesticides in the retention times (fig. 2.). In the figure 2 the chromatograms from the blank material and standard solution are compared by overlaid. The retention time of each of compounds is noted in the chromatogram. HCB was the first detected pesticide with retention time 18.20 minute and endosulfan sulfate was detected on the 36.77 minute. Internal standard Mirex was with retention time on 39.49 minute. The present study suggested including of internal standard Mirex to achieve improved chromatographic conditions.

To confirm that the developed method is suitable for its intended use, and to fulfill the aim of this study, the validation process was carried out. The quantification of OCPs was obtained using matrix calibration curve with internal standard. In the table I are presented results from validation based on spiked blank material at three concentration levels, as each concentration level were analyzed in three series, each in different day, and each in six replicates. Linearity, recovery, repeatability, reproducibility and uncertainty were evaluated for each of 15 OCPs. All 15 OCPs were qualitative determined in spiked blank samples, as the results from all replications were averaged. Regression coefficients (R2) values varied from 0.9957 to 0.9820, as the highest R2 was obtained for DDT, and the lowest was for γ-HCH. The recoveries of the individual compounds varied from 55.5 to 89.9%, as the lowest and highest were for HCB and β-HCH, respectively. Most of compounds obtained results near to 70 % priority in first validation level. Within-laboratory reproducibility, expressed as coefficients of variation (CV) was with highest value of 16.7 in the result for HCB.

Results from quantitatively determination of each OCPs was expressed as limit of quantification, recovery and repeatability calculated at LOQ-level and presented in table II. Limits of quantification for nearly all components is 0.025 µg/g fat, except for γ-HCH where it is 0.005 µg/g. In the table II are given also the legislative values for MRLs of each OCPs residue. LOQ reached by the developed method for DDT is 0.025 µg/g fat which is 40 times lower than MRL. The recoveries of the individual pesticide residues compounds varied from 53.5 to 87.8%. The lowest was for γ-HCH and the highest for DDD.

Discussion

European regulations required an increasing number of matrices and compounds to be included in the monitoring programs for control of pesticide residues based on effective methods with high level of quality control at the same time. Therefore, we have sought a new sample preparation procedure which allows seven additional pesticides (dieldrin, endosulfan- α and β isomers, endosulfan sulfate, HCB, heptachlor, heptachlorepoxide- cis isomer) to be included in multiresidue assay and to reach 15 of 17 OCPs named in EU regulations [8] and time and solvent consumption to be reduced. The method which had been used before for determination of OCPs residues in meat in Bulgarian

![Figure 2: Overlay chromatograms of blank material (fat extracted from meat) and multi-standard solution at the area of OCPs compounds](image-url)
national reference laboratory was based on EN 1528:2001 [3] but covers only 8 pesticides - aldrin, heptachlorepoxide-trans isomer, HCH – α, β, γ isomers, p,p'- DDE, p,p'- DDD and p,p'- DDT. It include hot solvent extraction using Soxtec extraction system with petroleum as solvent, followed by clean-up on florisil column and final pesticide determination by GC-ECD. Aiming to meet the European requirements for mandatory monitoring of pesticide residues in food of animal origin, the analytical scope of official control method needs to be extended and chromatographic conditions have to be improved and sample preparation have to be changed. The challenge in the new method development was that some of the newly added OCPs have very low MRLs, and for others it was impossible to be extracted from the lipid fraction via conventionally used florisil column.

Lipophilic pesticides as OCPs and lipids are extracted together from the meat samples by organic solvents, and analyzed residues are associated with the fat portion of the samples [3]. Methods for extraction of fat which are recommended should be simultaneously applicable for extraction and quantitatively determination of fat content and the residues in one sample preparation [3, 10]. Some of the most popular extraction techniques used in the laboratories for pesticides residues control of food of animal origin are Soxhlet and an improved version of Soxtec [4]. The disadvantage they have is that they require large amount of solvents, usually more than 300 ml, and thus the process takes hours to complete. Time and solvents consumption in the present method was reduced by application of accelerated solvent extraction technique (ASE) instead of classic Soxtec fat extraction. The combination of high pressure of 1500 psi and 100°C temperature in ASE allowed our method to reduce extraction process to about 15 min and to reduce solvent consumption up to 135 ml of per 10 g sample.

The other significant step in the developed method is replacement of florisil column clean-up step (up to 0.1 g capacity for fat purification in florisil method) with the liquid-liquid partitioning (0.5 g or more capacity for fat purification), which removes high fat content in sample extracts followed by purification step using homemade mini-silica column. Sample extracts in OCPs residues assay are with high fat content and must be involved in a process of lipid removal, purification and concentration. Insufficient removal of lipids and other co-extracts will reduce method

<table>
<thead>
<tr>
<th>R (EC) 396/2005 Pesticide EU MRLs for meat [µg/g fat]</th>
<th>Analyzed compounds</th>
<th>LOQ [µg/g fat]</th>
<th>Recovery [%], (n=20)</th>
<th>Repeatability RSDr [%], (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 Hexachlorobenzene</td>
<td>HCB</td>
<td>0.025</td>
<td>58.7</td>
<td>14.4</td>
</tr>
<tr>
<td>0.2 Hexachlorocyclohexane- α isomer</td>
<td>α-HCH</td>
<td>0.025</td>
<td>54.5</td>
<td>9.3</td>
</tr>
<tr>
<td>0.02 Hexachlorocyclohexane- γ isomer</td>
<td>γ-HCH</td>
<td>0.005</td>
<td>53.5</td>
<td>7.7</td>
</tr>
<tr>
<td>0.1 Hexachlorocyclohexane- β isomer</td>
<td>β-HCH</td>
<td>0.025</td>
<td>81.9</td>
<td>5.8</td>
</tr>
<tr>
<td>0.2 Aldrin and dieldrin(Aldrin and Dieldrincombined expressed as dieldrin)</td>
<td>Aldrin</td>
<td>0.025</td>
<td>56.7</td>
<td>11.3</td>
</tr>
<tr>
<td></td>
<td>Dieldrin</td>
<td>0.025</td>
<td>65.6</td>
<td>14.9</td>
</tr>
<tr>
<td>0.2 Heptachlor (sum of Heptachlor and Heptachlor epoxide expressed as heptachlor)</td>
<td>Heptachlor</td>
<td>0.025</td>
<td>59.1</td>
<td>13.6</td>
</tr>
<tr>
<td></td>
<td>HCE-cis</td>
<td>0.025</td>
<td>68.7</td>
<td>7.4</td>
</tr>
<tr>
<td>1 DDT (sum of p,p’- DDT, o p’- DDT, p,p’- DDE and p,p’- DDD expressed as DDT)</td>
<td>HCE-trans</td>
<td>0.025</td>
<td>69.8</td>
<td>12.5</td>
</tr>
<tr>
<td></td>
<td>p,p’-DDE</td>
<td>0.025</td>
<td>74.4</td>
<td>9.8</td>
</tr>
<tr>
<td></td>
<td>p,p’-DDD</td>
<td>0.025</td>
<td>87.8</td>
<td>13.5</td>
</tr>
<tr>
<td></td>
<td>p,p’-DDT</td>
<td>0.025</td>
<td>67.6</td>
<td>7.6</td>
</tr>
<tr>
<td>0.05 Endosulfan (sum of α and β-isomers and endosulfansulfate expresses as Endosulfan)</td>
<td>α-Endosulfan</td>
<td>0.025</td>
<td>67.1</td>
<td>12.2</td>
</tr>
<tr>
<td></td>
<td>β-Endosulfan</td>
<td>0.025</td>
<td>68.8</td>
<td>14.1</td>
</tr>
<tr>
<td></td>
<td>Endosulfansulfate</td>
<td>0.025</td>
<td>72.0</td>
<td>9.9</td>
</tr>
</tbody>
</table>

Table II: MRLs for OCPs in meat according to Regulation (EC) 396/2005 and validation parameters obtained in spiked blank material (fat extracted from meat) at the limit of quantification level (LOQ)
sensitivity and could damage the GC-column or detector. Various techniques for lipid removal are cited in the literature, such as Gel permeation chromatography (GPC), adsorption chromatography (alumina, silica-gel, florisil column), liquid-liquid partitioning with various organic solvents. In the recent years QuEChERS goes also popular in routine tests. The GPC clean-up is recommended as a technique with precise efficiency for the lipid removal, applicable to purify fat-rich extracts, which is automated and with good recovery results, but special maintenance required and increased time and solvent consumption [4, 15]. Adsorption chromatography using alumina, silica gel and florisil, or a combination of these materials is in use in food laboratories, but it cannot guarantee sufficient lipid purification of extracts with high fat content such as extracts from food of animal origin. Other technique is QuEChERS (used for extraction and clean-up steps) cited as a quick, easy, cheap, effective, but it has been applied with success on several non-fatty and low-fatty food matrices. Recently modified QuEChERS methodology has been applied in high fatty food as meat and fish, with additional clean-up step or liquid-liquid partitioning [6, 21].

The liquid-liquid partition, proposed in this paper, is simple and do not require special laboratory equipment and also makes the handling with the sample extracts very easy. Liquid-liquid partition is appropriate method for fat content removal [3], but additional clean step is needed because of possible precipitation of lipid traces in the defatted extract [6, 21]. Combination of liquid-liquid partition and silica gel column was used in our attempts. There are several of solvents used for liquid-liquid partitioning described in the literature. We made experiment with two mixtures to choose which is more suitable for liquid-liquid partitioning in our method. The tested mixtures were acetonitrile: hexane 1:1 (v/v) and acetonitrile: acetone 1:1 (v/v). Combination of acetonitrile: hexane was preferred because of the better separation between the two solvents layers and the easy discarding of the bottom hexane layer after partitioning. Actually, acetonitrile is polar enough to minimize the amount of co-extractive fat amount and it is non-polar enough to extract with efficiency lipophilic pesticides from the matrix [6]. During acetonitrile: hexane partitioning nearly all lipids remains into the hexane layer and are successfully eliminated with final hexane layer discarding. We tested also and two additional clean up steps: silica gel column clean-up and QuEChERS technique. Both techniques proposed good purification but results of QuEChERS (composed by 100 mg PSA, 400 mg Na₂SO₄, 100 mg C₁₈) showed less than 35% recovery for endosulfan-α and β isomers and less than 50 % recovery for aldrin, hexachlorocyclohexane (HCH) isomers - α, β and γ (lindane), hexachlorobenzene (HCB) (data not shown). Clean-up with laboratory homemade silica-gel column obtained better recovery of all 15 analytical compounds targeted by the new method (table I).

The calculation methodology using internal standard and calibration curve with spike samples is much more reliable and compensate recovery calculation in the routine work. The present study suggested including of internal standard Mirex to achieve improved chromatographic conditions. Analysis of pesticide residues was carried out with a gas chromatograph system (Trace-GC, Thermo, USA) and electron capture detector (ECD). The calibration curve showed very good linearity which assures applicability of used chromatographic system GC-ECD for the investigated 15 OCPs. The linearity of calibration curve for all pesticides was excellent with regression coefficients (R²) values varying from 0.9957 to 0.9820, as the highest R² was obtained for HCE-trans isomer, and the lowest was for γ-HCH (table I). Estimated validation parameters of the developed method were acceptable. The recoveries of the individual pesticide residues compounds varied from 55.5 to 89.9%. The lowest was for HCB and the highest for β-HCH. In the most single compounds residue analysis the recovery should be within the range from 70% to 120% in the first validation level. International guidelines for validation SANTE/11945/2015 stated that “in certain cases and typically with multiresidue methods, recoveries outside the range 70-120% may be accepted” and with an associated repeatability RSDr≤ 20% [23]. Exceptionally, where recovery is low but consistent (that is, demonstrating good precision) and when the basis for this is well established (for example, due to analyte distribution in a partitioning step), a mean recovery below 70% may be acceptable”. In our method the recovery is low, but consistent in the all 6 replicates on the 3 different days, which proof the acceptability of results. Other supporting argument is coefficient of repeatability of measurements and within-laboratory reproducibility, expressed as coefficients of variation (CV), which for our method were lower than 11.4% and 16.7% respectively. It is a very good result for multiresidue pesticide method.

The other parameter for evaluation of the new method is the lowest threshold for the limit of quantification. The regal requirement for acceptable value for LOQ is stated on SANTE/11945/2015 [23], where the LOQ must be lower than MRL. In our method the LOQ was determined as the lowest concentration level with acceptable values for recovery and precision. The maximum residues levels for all 15 OCPs analysed here are listed in the Regulation 369/2005 [22], and their values are summarized in table II. For all pesticide residues compounds (except γ-HCH) we set 0.025 μg/g fat as the lowest acceptable level for LOQ. For γ-HCH the threshold was set at 0.005 μg/g fat as acceptable level for LOQ. Comparing with the MRLs food safety requirements for Europe the achievement accumulated for LOQ by our method is 40 times lower than MRL for DDT, 8 times lower for HCB, α-HCH, aldrin, dieledrin, heptachlor and heptachlor isomers, 4 time lower for β-HCH, γ-HCH and only 2 times lower for endosulfan. That means that the achieved LOQ is considerable better than the 2 times required from the food laboratories when analyzing compounds with MRLs. The recoveries at the LOQ of the individual pesticide residues compounds varied from 53.5 to 87.8%. The lowest was for γ-HCB and the highest for DDD. Repeatability is in the range 5.8-14.9%.
METHOD FOR DETERMINATION OF ORGANOCHLORINE PESTICIDES IN MEAT

The new developed method allows OCPs pesticides to be successfully cleaned up and all of them to be available in the final extract with the yield sufficient for less than twice of established MRLs. Thus, the obtained analytical parameters meet the validation criteria. Our laboratory participates in regular proficiency tests for control of quality according to ISO 17025. We have successfully participated in proficiency test for lard-pig fat with our new method. The proficiency test has been organized by the European Union reference laboratory for pesticides in food of animal origin, Freiburg, Germany in 2016. The OCPs included in proficiency tests were HCH isomers - β (Z-score of result: 0.1), p,p'- DDE (Z-score of result: -0.6), endosulfan sulfate (Z-score of result: 0.5). The satisfactory z-score results confirm the success of the method development and validation.

In conclusion, the presented method covers the performance characteristics necessary for analysis of pesticides in meat and meat products. The proposed techniques for extraction and clean up are fast, cheap, easy and reduce the total amount of solvent used. The sample preparation effectiveness and the obtained validation parameters, contribute for the improvement of the analytical characteristics and extending the scope, which makes the method reliable and suitable for routine analyses in accredited laboratory.

Acknowledgements

The authors would like to thank to Central laboratory of veterinary control and ecology for the opportunity to use their specific equipment.

References

3. BDS EN 1528-1:2001 Fatty food – Determination of pesticides and polychlorinated biphenyls (PCBs), Bulgarian Institute for Standardization.
8. COMMISSION IMPLEMENTING REGULATION (EU) 2016/662 of 1 April 2016 concerning a coordinated multiannual control programme of the Union for 2017, 2018 and 2019 to ensure compliance with maximum residue levels of pesticides and to assess the consumer exposure to pesticide residues in and on food of plant and animal. Official Journal of the European Union L115, 2.


