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Validation and application of a method for determination of multi-class pesticides in muscle chicken breast fillets using QuEChERS extraction and GC/MS

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Abstract

Introduction: The occurrence of pesticide residues in animal products deserves attention because of the contamination by environmental pollutants and pesticides that may be present in the food that animals are fed. The goal of this work was the validation of a method for detection of residues of multiple classes of pesticide and determination of their residues in chicken breast fillets. **Material and Methods:** Gas chromatography with mass spectrometry was used for analysis. A modified quick, easy, cheap, effective, rugged and safe (QuEChERS) method was put into practice for its validation and applied to real samples. The study optimised mass detection and investigated the effect of a freezing step during the preparation of samples. Pesticides were determined in samples from conventional and organic production. **Results:** The impact of the matrix effect decreased, with the largest number of pesticides and satisfactory recovery determined by the application of mixed solvent acetonitrile and ethyl acetate for extraction. Detection of pesticide residues was achieved in a linear range between 5 and 50 µg/kg with satisfactory excellent correlation coefficients greater than 0.99. The recovery of all the pesticide residues ranged between 71.2 and 118.80%. The relative standard deviation was from 2.9% to 18.1% for all validated pesticide residues. The limits of quantification were in the range of 3.0–4.9 µg/kg. Out of 56 pesticide residues analysed in real samples, 5 were detected: α endosulfan, cypermethrin, endosulfan sulphate, permethrin and *p,p´*-dichlorodiphenyltrichloroethane (DDT) and their concentrations ranged from 4.9 to 15.2 µg/kg. **Conclusion:** All tested samples were compliant with the evaluation criteria, and detected values of pesticide residues were lower than the maximum residual levels.

Keywords: residues, chromatography, chicken meat, breast fillets.

Introduction

Cereals are the main component of animal feed for chickens. Among its cereal or cereal-derived components are oil products, grain mill products, corn, soybeans, starch products and dried plant products. Nowadays, growing cereals requires the use of a wide range of insecticides, fungicides and herbicides. The use of pesticides to prevent crop disease has increased significantly in recent years (21). Although the use of pesticides brings many benefits to agriculture, their residues can appear in poultry tissue and poultry products and some can affect human and animal health (15). Short-term exposure to pesticides does not have a significant harmful effect, while long-term exposure to certain pesticides can cause malignant diseases, cardiovascular diseases and damage to vital organs (5). Certain types of pesticide and their formulations may be used in different stages of cereal crop cultivation and during storage and can thus contaminate underground and surface water (6). Consequently, pesticide residues can be transferred to all compartments of the environment and thus reach poultry meat (12). Contamination of poultry may result both from direct exposure to pesticides in feed and water and from indirect exposure through environmental contamination. Chickens are also exposed to pesticides by inhaling them through contaminated air. Recently, much attention has been given to the importance of monitoring meat for the presence of pesticides. While many pesticide residues

cannot be fully metabolised in livestock and poultry, or are excreted in their original form in feces, certain residues remain and can be detected in tissue and fat. The occurrence of pesticides in the fat tissues of goats, sheep and cattle has been observed, where deltamethrin in sheep samples and hexachlorobenzene in cattle samples were present above the limit of quantification (11). In recent years, there has been a large number of documented detection of residues in chicken meat samples (20, 23, 29).

Poultry meat is the main protein source and the poultry industry is expanding in Serbia every year. Because of possible contamination, which is a general global concern around meat and animal products for both human and animal health, residue surveillance is necessary. Chicken is a good and cheap source of proteins, vitamins and minerals and contains a low amount of fat (3 g of fat per 100 g) compared to red meat which contains more than 5 g per 100 g. Chicken meat is a staple in the local diet, and analysis and monitoring of pesticide residues in this food product are necessary to ensure the safety of consumers. Every year, the European Food Safety Authority (EFSA) provides an annual report on the number of samples tested for the presence of pesticides in Europe (19). The report covers the type of food tested, the frequency of occurrence of pesticides in food, and the type and number of pesticides detected. Safety concerns have led to the establishment of maximum residue limits (MRLs) for pesticide residues in different food test matrix categories. Maximum residue levels are based on a risk assessment derived from pesticide residue data from food monitoring and food consumption data. Values of MRLs are applied to 315 fresh products and to the same products after processing, adjusted to content for dilution or concentration during the process. The general recommended value for the maximum residue level of pesticide contamination is 0.01 mg/kg and covers types of food for which values of pesticide contamination are not specifically defined (10).

The low limits of detection required by regulatory inspections and the complex nature of the matrix being tested for the presence of pesticides require efficient sample preparation and identification of traces of pesticides at the level of maximum residue values (12). The development of a multi-residue method is difficult, because pesticides have different polarities, solubility, volatility and pKa values (4). Over time, the amount of pesticides present can decrease because of their physical/chemical properties and environmental factors. The stability of pesticides can be influenced by the type of matrix, *i.e.* its composition, the presence of organic matter, pH influence and water content (3). Analysis of pesticide residues in fatty foods is a major challenge for analytical chemists, mostly due to the presence of water, fatty acids and cholesterol that make determination difficult. Pesticide residues can be analysed using gas chromatography with electron capture detection (GC/ECD) and gas (GC/MS) or liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) (13, 17). Most of the published multi-year studies for the analysis of pesticides in food of animal origin were carried out using gas chromatography/flame ionization detection (GC/FID) and the determination of persistent organic pollutants, *i.e.* organochlorine pesticides (2), but the scope of recent studies where, in addition to organochlorine pesticides, organophosphate pesticides, pyrethroids and carbamates are monitored, is proving to be more and more justified (23).

In the last few years, many studies have been published with the aim of developing sensitive and accurate methods for the determination of pesticides in animal matrices (28, 29). There is no unique standard preparation method using the quick, easy, cheap, effective, rugged and safe (QuEChERS) extraction procedure for matrices such as meat, fish and other fatty samples, but in scientific works and EFSA validations there is an increasing number of modified methods that include existing QuEChERS preparations for plant products (1, 4). Standard procedures for extraction of food of animal origin require the use of large quantities of reagents and solvents, many of which are toxic. They have several stages besides filtering, high-volume quantitative transfers and need the use of suitable laboratory dishes, evaporation and condensation steps, as well as annealing and activation of florisil for column cleanup. This complexity is highly relevant because every extra step makes the process more difficult and increases the risk of random and systematic mistakes (26).

The QuEChERS sample preparation method is a fast and modifiable method that was introduced in 2003 for rapid and efficient analysis of pesticide residues in products of plant origin by Anastassiades and Lehotay (1), and has been succesfully modified and improved by many other authors (21, 26). Thus, this method of dispersive solid phase extraction and cleanup has become the primary one in advanced analysis and simultaneous determination of a large number of pesticides, offering the possibility of modifying the method and adjusting the preparation technique for testing specific targeted pesticides. After two decades, this modern method of sample preparation has become the basic method and most rational choice. The use of QuEChERS for the preparation of samples for pesticide analysis has expanded over the years to also include fatty foods as possible samples (27), *i.e.* products of animal origin (meat, eggs, milk, *etc*.). QuEChERS has replaced the European standard method certified by the European Committee for Standardization (EN1528) in many laboratories because of the short duration of QuEChERS analyses and the method's use of less of particular expensive solvents. New sorbent materials with different types of adsorbent and different packages and ratios of composition quantities have become the basis of a new technology for testing the presence of pesticides. The components of dispersive solid phase consist of magnesium sulphate, C18 silica, primary-secondary

amine (PSA), graphite carbon black, Florisil, Z sep sorbent and a specially designed enhanced matrix removal lipid. The analysis of pesticides in food requires sample preparation, where precise separation and detection is important. The preparation techniques for analysing pesticides from the sample are: liquid-liquid extraction, liquid-liquid micro extraction, solid-phase extraction (SPE), solid-phase micro extraction and gel-permeable chromatography. Solid-phase extraction is currently the best represented and best used extraction technique (29). The first step in pesticide analysis is the choice of method and the second step is the validation of the precise method and optimisation of the technique on the instrument on which the determination is performed.

These steps are preparatory work for every comprehensive monitoring programme conducted to verify compliance with legislation; such programmes are essential for realistic assessments of exposure to dietary chemicals. The use of a modified QuEChERS method for monitoring different matrices marks significant progress in assessing food safety, but validated QuEChERS methods need to be confirmed through proficiency testing (PT) activities and accreditation under ISO/IEC 17025. The aim of the study is primarily to summarise an instance of progress in research on gas chromatographical quantification of pesticide residues in chicken fillets and to confirm the importance of multi-class analysis. The validated method was applied to test pesticides in conventional and organic muscle chicken breast fillets.

Material and Methods

Chemicals and reagents. Highly pure analytical standards of pesticides residues were obtained from Dr. Ehrenstorfer (Augsburg, Germany). Stock standard solutions of investigated pesticides (10 µg/mL) were prepared by dissolving 1 mL of each standard mix solution in 10 mL of acetonitrile in a volumetric flask. The prepared solutions were stored in a dark place at −20°C for no longer than 3 months. Calibration standards at concentrations of 5, 10, 20, 30 and 50 µg/kg were prepared by serial dilution using hexane as a solvent. Pesticides that can simultaneously or individually act as acaricides, insecticides, nematicides, fungicides and plant growth regulators were tested. These pesticides belong to these chemical classes: organochlorines, organophosphates, carbamates and pyrethroids. Analysis was undertaken of residues of acephate, aldrin, α-endosulfan, α-hexachlorocyclohexane (HCH), azoxystrobin, β-endosulfan, β-HCH, bifenthrin, boscalid, carbaryl, carbofuran, chlorfenapyr, chlorpyrifos, chlordane (*cis*- and *trans*), cyfluthrin (sum of isomers), cypermethrin (sum of isomers), δ-HCH, diazinon, dichlorvos, dieldrin, dimethoate, endosulfan sulphate, endrin, endrin ketone, etofenprox, ethoprophos, etoxazole, fenoxycarb, fipronil, fipronil sulfone, fludioxonil, heptachlor, heptachlor epoxide (*trans*-, isomer A), imazalil, kresoxim-methyl, lindane, malathion, metalaxyl, methyl parathion, methoxychlor, ortho (*o*) and para (*p*)′-dichlorodiphenyltrichloroethane (DDT), *p*,*p*′- dichlorodiphenyldichloroethane (DDD), *p*,*p*′- dichlorodiphenyldichloroethylene (DDE), *p*,*p*′-DDT, paclobutrazol, permethrin, phosmet, prallethrin (*cis*- and *trans*), propiconazole, propoxur, pyridaben, spiromesifen, spiroxamine, tebuconazole and trifloxystrobin. Triphenyl phosphate purchased from Dr. Ehrenstorfer was used as an internal standard (INSTD) to spike sample extracts before extraction. A stock solution of triphenyl phosphate at a concentration of 1,000 µg/mL was prepared by dissolving of 10 mg of INSTD in 10 mL of toluene in a volumetric flask. A working solution of INSTD at 10 µg/mL in hexane was prepared for all extractions and for all sample analyses. Acetonitrile, ethyl acetate, toluene and hexane were obtained from J.T. Baker (Deventer, the Netherlands). Ultra-pure water (18.2 MΩ/cm) was generated in house using a WP4100 apparatus (Smeg Instruments, Guastalla, Italy). The QuEChERS extraction agents were manufactured by Agilent (Santa Clara, CA, US).

Sampling. A total of 100 commercial samples of chilled chicken fillets were purchased from the local market in Belgrade (Serbia). Before analysis, the samples were refrigerated at temperatures between 0 and 4°C. There were 40 samples of chicken fillet from domestic producers, 40 samples of imported chicken and 20 samples of domestic production produced and labelled as organic products. The chicken fillets were taken for analysis in the period from March to June 2023. The samples were packed in polyethylene bags, labelled, placed on ice and delivered to the laboratory. The samples were then stored in a refrigerator at 2°C until initiation preparation for analysis. All purchased samples were of 1 kg mass.

Extraction and clean-up steps. All chicken breast muscle fillet samples were prepared using a homogeniser (IKA-Werke, Staufen, Germany). Portions of the homogenised samples were taken for further extraction and placed in 50 mL polyethylene tubes until the mass of sample was 10 g. Pesticide extraction and cleanup steps in preparation of samples were performed according to published procedures (1, 21, 27), with modifications. The method was modified in the extraction step with the use of a mixture of extraction solvents instead of acetonitrile. Aliquots of 10 mL of mixtures of solvents (acetonitrile:ethyl acetate, 1:1 (v/v) were added and the mixture was shaken using a vortex mixer. Before extraction, 100 µL of INSTD at 10 µg/mL was added to the 50 mL polyethylene tubes. The samples were shaken by an LBX Instruments V03 series Mini Vortex Stirrer (Labbox Labware, S.L. Barcelona, Spain) for 20 s prior to having the QuEChERS reagent added. More precisely, 4.0 g of anhydrous magnesium sulphate and 1.0 g of sodium chloride (Agilent) were added, then extraction was undertaken by shaking the mixture vigorously on the vortexer for 2 min and spinning it for 5 min at 4,000 rpm on a EBA 280 centrifuge (Hettich,

Beverly, MA, US). An aliquot of 8 mL was transferred from the supernatant to a new, clean 15 mL centrifuge tube containing the sorbent, 150 mg of PSA, 150 mg of C18 and 900 mg of anhydrous magnesium sulphate, all components of a d-SPE QuEChERS kit (Agilent) in a simple approach termed dispersive solid-phase extraction (d-SPE) cleanup. The samples were again shaken on the vortex mixer for 3 min, frozen out at −20°C for half an hour and then centrifuged for 5 min at 4,000 rpm. A Bibby Scientific sample concentrator (Stone, UK) was used for the concentration step of the extracts. An aliquot of the supernatant was evaporated to dryness under a stream of nitrogen, dissolved in hexane and injected into a gas chromatograph.

Method validation. The validation procedure was based on the one prescribed by the Directorate-General for Health and Food Safety of the European Commission in the SANTE/11312/2021 document (9). A blank meat sample (Fapas, Sand Hutton, UK) was prepared for calibration as quality control material in order to monitor the influence of the matrix and the calculation of recovery parameters. The extraction and cleanup phases were carried out on this sample in the same way as on the other samples for analysis. After evaporating the extract to dryness and reconstituting it, an internal standard in a concentration of 100 µg/kg and calibration standards in the range from 5 to 50 µg/kg were added. Matrix calibration standards were used to construct matrix calibration curves. For estimation of recovery and precision, which is indicated by relative standard deviation (RSD), 10 g of a blank sample of chicken meat was spiked with a pesticide standard mix at 5, 10 and 20 µg/kg at the beginning of the extraction and this was repeated in five replicates for each standard. During the quantification of detected pesticides in real samples, each sequence contained spiked blanks and verification standards at the lowest and highest concentrations. The prepared samples were analysed with mass spectrometry in the selected ion monitoring (SIM) mode using one target and three qualifier ions for each analyte. The ratio of the qualifier to the quantifier ions in the sample was compared to the ratio in the standard for the same retention time. Validation parameters and calibration characteristics data are presented in Supplementary Table S1.

Gas chromatography and mass spectrometry analysis instrumentation and parameters. Pesticide residues were analysed using a Clarus 680 Gas Chromatograph system (PerkinElmer, Waltham, MA, USA) and a Clarus SQ8T mass spectrometer (PerkinElmer). The injector operated at the temperature of 250°C. An Elite-5MS capillary column of, 30 m length, 0.25 mm internal diameter and 0.25 µm film thickness with 5% phenyl and 95% dimethyl polysiloxane was used for the separation of the target components. Helium of 99.999% purity was the carrier gas was used at the constant pressure of 18.5 psi. The initial oven temperature of 60°C was held for 4 min and then increased to 145°C at 25°C/min, then increased to 210°C at 3°C/min, and further increased to 280°C at 8°C/min and held for 10 min, resulting in a run time of 42 min. The solvent delay time was 5.0 min. The injection volume was 2.0 µL in spitless mode. The mass spectrometer (MS) inlet line and the ion source temperatures were set at 280°C and 250°C, respectively, and the MS ionisation energy was 70 eV. The SIM mode was set for all determined residue pesticides in appropriate time intervals, keeping the dwell time of 0.020 ms. The obtained data was acquired by TurboMass software, version 6.1.0 (PerkinElmer).

Matrix effect. Matrix effect (ME) was calculated according to the formula published by Kumar *et al.* (16):

$$\%ME = \left(\frac{Slope_{matrix-matched}}{Slope_{solvent}} - 1\right) \cdot 100\%$$

The ratio of the slope of the matrix-matched blank extract calibration curves to the slope of the calibration in solvent was used for the calculation of ME. The method performance in respect of matrix effect characteristics is presented in Table 1. Matrix calibrations are compared with calibration in solvent, where, according to other authors and the SANTE/11312/2021 guideline, the tolerance is ±20%. Percentages of ME less than zero (*i.e.* negative value) indicate matrix suppression, while those greater than zero (*i.e.* positive value) indicate matrix enhancement. To avoid ME influence and possible quantification errors, matrix-matched calibration curves were used for quantification and compensation of the matrix effects.

Conversion factors. Product safety monitoring is under the legal regulation to include the expression of MRL as the sum of isomers, metabolites and/or transformation products. To comply with this and for harmonising the obtained results with the defined MRL value, it was necessary to incorporate conversion factors, the calculation of which is specified by the SANTE guidelines (9). They set out calculations based on different molecular weights of existing functional groups.

The following conversion factors were used to calculate the tested pesticides:

$C_{\text{(sum of dieldrin and aldrin)}} = 1.00 \times C_{\text{dieldrin}} + 1.053 \times C_{\text{aldrin}}$

$C_{\text{DDX}} = 1.00 \times C_{p,p'\text{-DDT}} + 1.00 \times C_{o,p'\text{-DDT}} + 1.114 \times C_{p,p'\text{-DDE}} + 1.107 \times C_{p,p'\text{-DDD}}$

$C_{\text{total endosulfan}} = 1.00 \times C_{\text{alpha-endosulfan}} + 1.00 \times C_{\text{beta-endosulfan}} + 0.962 \times C_{\text{endosulfan sulphate}}$

$C_{\text{total heptachlor}} = 1.00 \times C_{\text{heptachlor}} + 0.958 \times C_{\text{heptachlor epoxide}}$

$C_{\text{total fipronil}} = 1.00 \times C_{\text{fipronil}} + 0.965 \times C_{\text{fipronil sulfone metabolite (MB46136)}}$

where C is the concentration of an individual quantified metabolite, which can be expressed in milligrams or micrograms per kilogram, and where the values in front of the concentrations are the obtained conversion factors (Cf) for the given metabolite. The Cf conversion coefficient is equal to one for determining the sum of *cis*- and *trans*- isomers.

Results

Optimisation of GC/MS method. In first analytical step, a GC/MS technique was developed for identification and quantification of 56 GC-amenable pesticide residues from different chemical classes. Under instrumental conditions on a GC-MS column, the standard with the highest concentration (50 μg/kg) for the tested pesticides applicable to GC analysis was used in scan mode to obtain their full scan spectra and retention times. The full scan mode was selected in the range *m/z* 40–450 for scanning monitoring ions, the quantifier ions and qualifier ions with the highest sensitivity (Table S1). During calibration and analysis, chromatograms were recorded in full scan and SIM mode. Calibration and quantification using the SIM mode negates more of the influence of co-extract interference and achieves a lower limit of quantification. The mass spectrum parameters were optimised to provide good chromatographic separation, identification and quantification of the developed method. Ions with more than 15% abundance compared to the quantitative base peak ions were selected for quantification for each compound.

The optimisation of the method of preparation was carried out experimentally by monitoring extraction with acetonitrile, extraction with acetyl acetate, and as a third option, extraction with a mixture of acetonitrile and ethyl acetate in a ratio of 1:1 (MeCN:EtOAc). Monitoring the degree of pesticide recovery, the best recovery with the highest percentage of satisfactory pesticides was achieved using MeCN:EtOAc. The use of a matrix-matched calibration solution was necessary to minimise errors associated with matrix-induced enhancement or suppression effects during GC determination. Blank chicken extracts were used for the elimination of the background noise during chromatographic determination. Calibration in chicken extract was also beneficial to the assessment of matrix effects. Blank matrices of chicken meat, representing the same matrix in which the presence of pesticides is tested and having water, fatty acid and cholesterol content, were used for the preparation of calibration standards in order to account for matrix effect. The lower chromatogram in Fig. 1 shows the influence of the matrix effect of the tested pesticides determined for chicken breast fillet using the original methods and acetonitrile as extraction solvent, while the upper figure shows a modification of the use of MeCN:EtOAc for extraction. A significantly smaller influence of the matrix effect and an absence of most of the cholesterol peak at a retention time of 36.26 min was observed using the solvent mixture (confirmed by the US National Institute of Science and Technology database).

Validation, linearity, specificity, limit of quantification and limit of detection. Method optimisation was carried out with consideration of the following validation parameters: stability, selectivity, linearity, precision, trueness, limit of detection (LOD), limit of quantification (LOQ) and robustness. The limit of quantification represents sensitivity to all GC-amenable compounds from the standard mixture of pesticides. The limit of detection and LOQ were determined based on the signal-to-noise ratio, and concentrations showing a peak signal-to-noise ratio intensity of 3 and 10 were designated as the LOD and LOQ, respectively. These parameters were determined from six replicates of the lowest concentration of the calibration curve (Table 1). Linearity was determined by calibrating the pesticide against an internal standard. Trueness was confirmed during recovery for sample pretreatment and matrix effect which improve chromatographic separation. Each analytical analysis included blank solvent, calibration verification standards, tested samples and spiked samples. The results for accuracy and precision are expressed for the three different concentration levels in relation to recovery and RSD (Table 1). The use of certified reference material confirmed the accuracy of the validation method within the limits of the used measurement uncertainty. The mean value of the result obtained by the validated method for α-HCH was 25 μg/kg, while the allowed value of certified reference material was 24.1 ± 7.2 μg/kg. The validation method performance characteristics are shown in Table S1. The results for samples spiked at the concentration levels 5 μg/kg, 10 μg/kg and 20 μg/kg are shown in Table 1. The validated method was confirmed through PT activity in May 2022 where the determination of fipronil and fipronil sulfone was performed on a sample provided by Fapas.

Analysis of retail chicken meat samples. The validated method was applied to detect and quantify residues of the pesticides in a hundred samples of frozen chicken fillet. The quantification of the investigated pesticides was achieved based on the calibration in the solvent because the influence of the matrix effect was in a range from 0% to ±15%, which is considered not to be any influence. Also, the influence of the ME was diminished when the acetonitrile and ethyl acetate extraction solvent mixture was used; the mixture extracted the largest number of pesticides with and the best satisfactory recovery. Quantification was performed based on the area of the quantifier ion according to the internal standard. Also, care was taken that the ions and their surfaces overlapped with the standard and met the prescribed tolerance range of the maximum retention time (±0.1 min) shown in Table S1. For the purposes of pesticide detection and analysis of multiple residues in chicken fillet, GC/MS combined with the QuEChERS technique was found to be effective. The obtained results for pesticides detected above the LOQ are shown in Table 2, and their values are less than the MRL sum, that is the maximum residue limits defined by EU regulations. The pesticides were detected in triplicate and also confirmed with the spiked sample at the lowest concentration level from the calibration curve.

Table 1. Recovery, relative standard deviation (RSD), limit of detection (LOD) and limit of quantification (LOQ) for determination of multi-class pesticides

No.	Pesticide	Recovery (RSD), % (n = 6) 5 µg/kg	Recovery (RSD), % (n = 6) 10 µg/kg	Recovery (RSD), % (n = 6) 20 µg/kg	LOD (n = 6) µg/kg	LOQ (n = 6) µg/kg
1	Acephate	72.8 (9.2)	71.8 (9.1)	84.2 (14.9)	1.1	3.6
2	Aldrin	75.2 (11.2)	78.2 (10.0)	105.2 (9.8)	1.3	4.2
3	α-Endosulfan	83.2 (6.5)	79.4 (7.0)	84.0 (9.8)	1.2	4.0
4	α-HCH	81.2 (6.1)	79.6 (9.4)	77.4 (6.3)	1.4	4.5
5	Azoxystrobin	100.5 (3.6)	103 (13.3)	82.1 (10.2)	1.0	3.3
6	β-Endosulfan	88.2 (4.2)	87.6 (13.3)	82.1 (10.2)	1.2	4.1
7	β-HCH	102.4 (11.3)	114.2 (14.4)	110.0 (14.8)	1.2	4.0
8	Bifenthrin	92.3 (8.2)	94.8 (10.9)	80.8 (11.3)	1.2	3.9
9	Boscalid	102.3 (4.6)	109.0 (10.9)	95.6 (10.7)	1.1	3.7
10	Carbaryl	102.4 (3.8)	95.6 (8.9)	106.2 (16.2)	1.5	4.9
11	Carbofuran	86.9 (11.9)	83.8 (13.6)	84.0 (10.1)	1.4	4.8
12	Chlorfenapyr	79.5 (5.2)	71.2 (11.7)	82.4 (12.6)	1.6	5.2
13	Chlorpyrifos	99.4 (3.2)	97.0 (6.1)	84.6 (12.0)	1.4	4.5
14	*cis*-Chlordane	90.7 (6.3)	90.8 (12.7)	92.0 (14.2)	1.4	4.5
15	Cyfluthrin (sum)	112.5 (7.2)	114.1 (12.9)	117.2 (7.8)	0.9	3.1
16	Cypermethrin (sum)	99.5 (3.5)	110.0 (9.6)	84.4 (15.5)	1.2	4.0
17	δ-HCH	93.4 (9.2)	89.8 (10.3)	110.2 (11.7)	1.1	3.7
18	Diazinon	99.9 (8.1)	96.3 (11.4)	105.8 (1.7)	1.4	4.6
19	Dichlorvos	83.2 (12.4)	82.2 (14.9)	83.2 (12.7)	1.5	4.9
20	Dieldrin	105.6 (4.5)	107.2 (6.2)	113.0 (14.3)	0.9	3.1
21	Dimethoate	111.1 (4.3)	112.2 (7.2)	112.6 (4.5)	1.0	3.2
22	Endosulfan sulphate	98.5 (5.8)	114.4 (4.1)	102.2 (8.3)	0.9	3.1
23	Endrin	87.8 (8.2)	82.8 (13.6)	73.6 (7.5)	1.0	3.5
24	Endrin ketone	82.5 (3.6)	81.0 (13.8)	101.6 (2.9)	1.0	3.4
25	Etofenprox	99.9 (11.6)	102.0 (18.9)	95.5 (13.2)	1.0	3.5
26	Ethoprophos	94.6 (5.7)	104.0 (7.8)	87.8 (14.3)	1.4	4.8
27	Etoxazole	111.6 (14.2)	118.8 (12.5)	113.0 (12.5)	1.4	4.7
28	Fenoxycarb	89.2 (7.8)	77.2 (13.5)	81.8 (17.8)	1.4	4.5
29	Fipronil	106.3 (12.4)	104.6 (17.4)	86.8 (17.9)	0.9	3.0
30	Fipronil sulfone	103.1 (3.5)	102.1 (6.3)	109.4 (4.9)	0.9	3.0
31	Fludioxonil	86.3 (4.9)	89.4 (4.6)	80.0 (18.1)	1.2	4.0
32	Heptachlor	85.4 (4.1)	80.2 (4.1)	86.0 (8.5)	1.2	4.0
33	Heptachlor epoxide (*trans*-, isomer A)	98.7 (4.4)	89.6 (11.8)	99.8 (15.2)	1.4	4.8
34	Imazalil	99.8 (12.3)	97.4 (16.6)	102 (18.0)	0.9	3.1
35	Kresoxim methyl	94.4 (2.9)	89.2 (7.8)	98.0 (3.6)	1.0	3.4
36	Lindane	79.3 (6.1)	77.4 (7.7)	75.4 (15.1)	1.2	4.0
37	Malathion	84.6 (6.9)	79.4 (15.4)	76.2 (8.1)	1.4	4.6
38	Metalaxyl	90.2 (4.3)	90.8 (11.5)	106.0 (7.6)	1.4	4.6
39	Methyl parathion	84.1 (6.7)	83.8 (13.8)	84.5 (6.1)	1.4	4.6
40	Metoxychlor	74.7 (4.2)	74.8 (6.8)	101.2 (16.7)	0.9	3.1
41	*o,p'*-DDT	92.6 (3.9)	95.6 (3.3)	97.9 (3.7)	1.2	4.0
42	*p,p'*-DDD (TDE)	100.1 (2.9)	89.4 (7.0)	101.2 (16.7)	1.2	4.0
43	*p,p'*-DDE	94.1 (1.6)	93.5 (3.5)	95.8 (11.8)	1.0	3.5
44	*p,p'*-DDT	94.2 (3.0)	84.8 (12.1)	101.0 (13.5)	1.0	3.5
45	Paclobutrazol	87.7 (3.5)	79.8 (5.7)	88.0 (13.7)	1.4	4.6
46	Permethrin (sum)	86.6 (8.9)	83.8 (14.8)	102.2 (8.4)	1.0	3.5
47	Phosmet	103.1 (2.9)	95.2 (14.1)	106.6 (10.6)	1.5	4.9
48	Prallethrin (sum)	98.5 (5.5)	102.4 (17.8)	92.6 (5.7)	1.0	3.3
49	Propiconazole (sum)	101.4 (11.3)	96.0 (10.6)	104.8 (14.4)	1.4	4.6
50	Propoxur	75.3 (3.1)	87.4 (10.9)	75.2 (9.7)	1.2	4.0
51	Pyridaben	110.3 (3.3)	114.0 (8.1)	85.4 (12.3)	1.4	4.8
52	Spiromesifen	89.9 (9.9)	81.6 (14.1)	90.8 (10.0)	1.2	4.0
53	Spiroxamine (sum)	113.4 (14.7)	118.8 (10.8)	105.4 (9.9)	1.4	4.6
54	Tebuconazole	94.2 (7.1)	110.6 (6.4)	71.2 (11.1)	1.2	4.0
55	*trans*-Chlordane	91.2 (4.6)	86.0 (10.3)	107.8 (17.2)	1.4	4.6
56	Trifloxystrobin	104.2 (2.9)	107.0 (15.1)	87.2 (3.0)	1.2	4.0

HCH – DDT – dichlorodiphenyltrichloroethane; DDD – dichlorodiphenyldichloroethane; DDE – dichlorodiphenyldichloroethylene

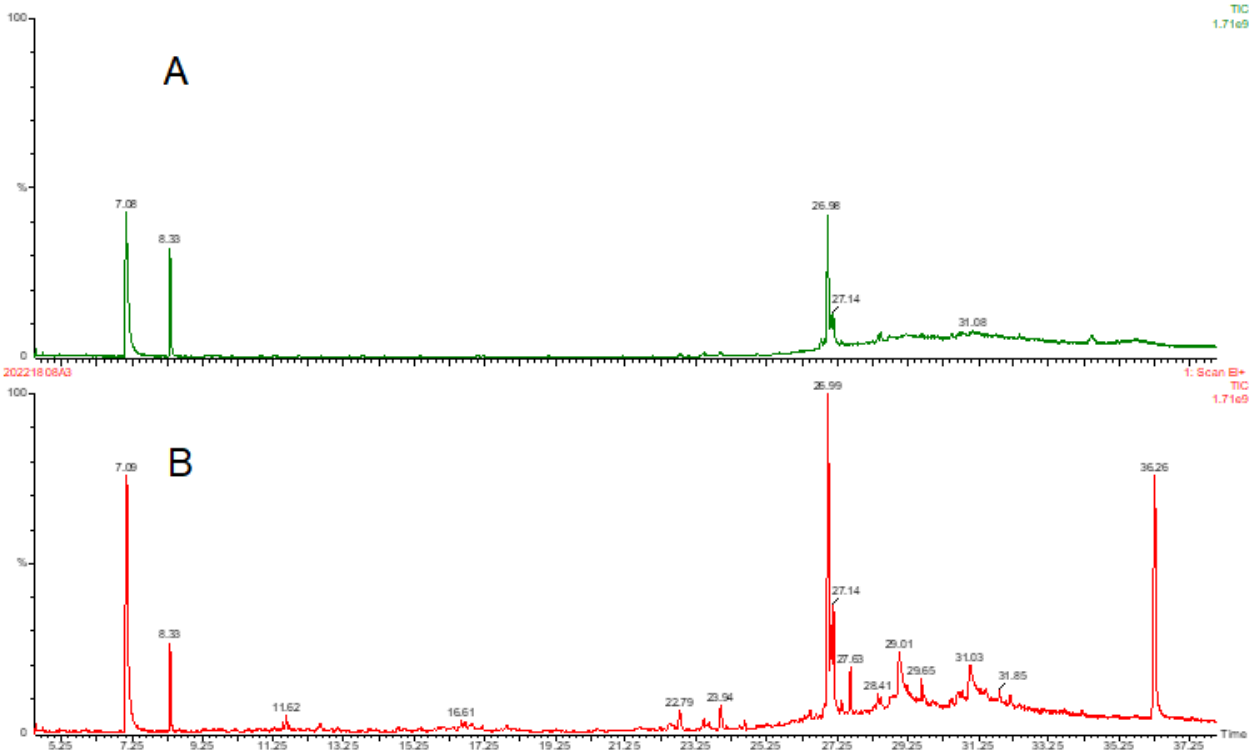

Fig. 1. Full scan gas chromatography and mass spectrometry chromatograms for the analysis of multi-class pesticides in method optimisation (A) using a mixture of acetonitrile and ethyl acetate as the extraction solvent, (B) using acetonitrile as the extraction solvent

Table 2. Pesticides detected and their concentrations in chicken breast fillets

Sample	Number of samples, pesticide <LOQ	Number of samples, pesticide, >LOQ	Cypermethrin (sum of isomers – µg/kg)	Endosulfan sulphate, (µg/kg)	Permethrin, (sum of isomers) µg/kg	Alpha endosulfan, µg/kg	*p,p´*-DDT, µg/kg
Domestic chicken fillets	37	3	<LOQ–11.5	<LOQ–4.9	<LOQ–15.2	<LOQ -5.4	<LOQ
Imported chicken fillets	39	1	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ–6.0
Organic domestic chicken fillets	20	0	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Calculated value using Cf	/	/	11.5	4.7	15.2	5.4	6.0
MRL (sum)	/	/	100	50	50	50	1000

LOQ – limit of quantification; DDT – dichlorodiphenyltrichloroethane; Cf – conversion factor; MRL – maximum residue limit

Discussion

Validation is always a balance between skills and technical possibilities. Validation using QuEChERS preparations avoids the use of large amounts of expensive organic solvents with the help of extremely powerful and highly sophisticated techniques. The validation guideline (SANTE/11312/2021) (9) prescribed by European regulations precisely defines the conditions for linearity, correlation coefficient, recovery, repeatability, accuracy and relative standard deviations. Following the steps prescribed by the guide allows the laboratories to have free choice of methods, which is beneficial for the continuous evaluation of the analytical methods. Laboratories performing analyses of pesticide residues also tend to work under a quality system and standard ISO/IEC 17025. In this study, LOD and LOQ values ranged 0.9–1.5 µg/kg and 3.0–4.9 µg/kg, respectively. The LOQ values of all studied pesticides were below their respective MRLs, which confirms that the validated method has satisfactory sensitivity. During the validation of the method, there were no deviations in recovery values within the range of 70–120%, and all obtained repeatability RSD values were less than 20% (Table 1). Acceptable recoveries were obtained for the tested pesticides. Satisfactory sensitivity was achieved and the correlation coefficients of all tested pesticides of the constructed calibration curves were greater than 0.99 (Table S1). When optimising the extraction procedure, solvents such as acetonitrile, methanol, acetone and ethyl acetate can be used as mixtures with water and/or additives for pH value adjustment (11, 16, 28). Therefore, differents amount of extraction agent can be used for the same mass of sample; freezing or ultrasound can also be

effective techniques (25, 28, 29). Rutkowska *et al.* (25) used 20 mL of acetonitrile and 10 mL of water, which was cold in order to reduce the endothermic reaction of magnesium sulphate and protect heat-sensitive pesticides such as captan, folpet, tolylfluanid, and dichlofluanid, when analysing 10g of commercial crop seed for residues. The composition of the matrix and target pesticides must be considered so that the preparation phase gives a satisfactory recovery and the influence of the matrix effect is minimised. The matrix effect is the significant influence of interfering substances from the sample matrix that is important to consider in both liquid and gas chromatography. The QuEChERS method grants the possibility of easy modification and adjustment of the preparation procedure for better reliability of testing specific or required pesticides in the tested matrix. Some modifications of the original QuEChERS method have been carried out to adapt it better to the matrix analysed and the characteristics of the analysed pesticides. By choosing appropriate sorbents for sample preparation, the influence of the matrix can be significantly reduced. Oliveira *et al*. (22) conducted a mixture design experiment on trahira (*Hoplias marabaricus*) samples to find an adequate combination of C18, PSA and Z-Sep+ with which to extract pyrethroids. The same experiment also investigated temperature treatment in sample preparation and achieved good results when a freezing time was chosen of 12 hours (overnight).

Sixty percent of the tested pesticides had a matrix effect value lower than 5%, 40% of them had a matrix effect value between 5 and 10%, and only pyridaben had a higher value of matrix effect, of 12.59%. The quantification was carried out in the matrix and the mean value of recovery was taken into account when quantifying the results. By examining the samples, the presence of pesticide residues was determined in four tested samples of chicken breast fillet. In 96.0% of the tested samples, none of the 56 tested multi-class pesticides were detected, while pesticide residues were detected in four samples, albeit at levels below the maximum residue levels (MRLs) values set by European regulation in 2005 (8, 10). This regulation was relevant to the present research in Belgrade because European Union legislation has been adopted and implemented in Serbian regulations regarding the use of pesticides and the presence of residues in food. In one sample from domestic producers, cypermethrin was detected in the amount of 11.5 µg/kg, which is less than the MRL value of 100 µg/kg (or 0.1 mg/kg). Also, in one sample from domestic producers, the persistent organochlorine pesticide endosulfan sulphate was detected in an amount of 4.9 µg/kg, while the MRL value is 50 µg/kg (0.05 mg/kg). Permethrin was detected in one sample from domestic producers in the amount of 15.2 µg/kg (MRL = 50 µg/kg) and endosulfan alpha was quantified in the same sample in the amount of 5.4 µg/kg (EU MRL value is 50 µg/kg). In a fourth sample, which was imported, the persistent pesticide *p,p´*-DDT was detected in an amount of 6 µg/kg, significantly less than the amount prescribed by law for the sum of all metabolites of DDT of 1,000 µg/kg. In our investigation, in addition to *p,p'*-DDT, the presence of the metabolite *p,p'*-DDE was confirmed in the amount of 3.2 µg/kg, but since the value is lower than the LOQ, it is not shown in Table 2. European Commission Regulation (EC) 2023/163 prescribes MRLs for DDT and its metabolites in various meat products at a maximum of 1,000 mg/kg. Dichlorodiphenyltrichloroethane is an active substance known as a persistent organic pollutant, and despite the cessation of the use of DDT in the European Union, it can still be detected at very low levels in some plant and animal products because it persists long in the environment. Based on the most recent data from 2016 to 2020, which show that DDT residues occur in wild boar products at levels higher than the current MRL, the Commission has established values for other farmed terrestrial animals at 1 mg/kg. For the pesticides for which MRLs are not prescribed, a default MRL of 0.01 mg/kg applies, and this is the reason for the quantification limits set in the SANTE guidelines, which must be achieved during method validation. Also, when using a lower calibration range from 5 to 50 µg/kg, validation can achieve lower LOD and LOQ values. Such lower values were also achieved by Kartalović *et al.* (14), whose LODs ranged from 0.27 to 1.51 µg/kg and LOQ values from 1.10 to 5.20 µg/kg. Using the conversion factor, there was a change in the value when calculating the total amount of endosulfan, whereby the amount of endosulfan sulfate (from 4.9 µg/kg) expressed per endosulfan was reduced to 4.7 µg/kg. All detected values are significantly lower than the EU MRL values and do not pose a risk to human or animal health. In the samples produced by organic production, none of the tested pesticides was detected. The organic samples had no traces of pesticides present. Good agricultural and animal husbandry practices led to chicken breast fillets farmed organically being pesticide residue free, which satisfies a requirement of organic production regulations. Above all, surveillance should not be omitted, because the EFSA recommended focusing monitoring activities on commodities that frequently contain pesticide residues or that have the potential to result in a significant short-term intake. The results obtained are in accordance with those of a study by Lee *et al*. (18), which examined 29 pesticides and their metabolites in 70 commercial chicken products from Korea. In their results, difenoconazole and propiconazole were detected in two samples and the concentrations were 11 µg/kg and 5 µg/kg. While they detected pesticides different to those found in our study, the detected amounts were in the same range as the one obtained by testing in our work. The presence of difenoconazole, imidacloprid and fipronil and its metabolites was confirmed by Wang *et al.* (28) examining chicken faeces using LC/MS/MS, which is important for validation and monitoring. Meat and meat products have also been evaluated in the neighboring Republic of Bosnia and Herzegovina: smoked pork products were examined and their concentrations of

α-HCH, lindane, PCB 28, PCB 52 and PCB 153 were quantified. The detected values were in the range of 5.1 ± 1.1 μg/kg for α-HCH and up to 22.11 ± 7.12 μg/kg for lindane, but the concentrations varied depending on the way the product was treated (14). Examinations of fish from the Serbian section of the Danube river are published more frequently in scientific literature. For example, the presence of *p,p'*-DDE and *p,p'*-DDD was confirmed in silver bream and barbel in the amount of 0.84 to 9.06 μg/g of fat (7). Considerable quantities of organochlorine pesticides in Serbia were quantified as metabolites of DDT, aldrin, lindane and endrin-ketone in amounts from 1.1 to 137.9 μg/kg in wild animals (24). The presence of pesticide residues in wild animals is explained by their presence in regions with intensive agricultural production and waste. However, there are no published articles on the topic of pesticides in chicken fillets in Serbia. In that regard, this study is the first of its kind.

Conclusion

The study presents a multi-class method for monitoring and controlling the presence of pesticide residues in chicken breast fillets. Sample preparation methods were validated and optimised for the analysis of pesticide residues in the chicken fillets using GC/MS for quantification. The determination of pesticides in chicken meat is necessary for ensuring that human exposure to possible contamination is minimal and is an important tool for the determination of environmental contamination by pesticide residues. Analysis of pesticides in fatty food such as poultry is a difficult and demanding laboratory task. Extraction and preparation of samples with satisfactory recovery is an important aspect of pesticide analysis which was achieved in this study. Quantification using sensitive instruments and the newest techniques, when reliable, is an important method for monitoring and screening of poultry meat. QuEChERS methods and means of monitoring which are suitable for determining traces of residues have led to modernisation and have shed more light on the actual levels of pesticide residues in food consumed on a daily basis. The development of fast, safe and efficient methods of preparation has made it possible to determine different classes of compounds.

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