Development and validation of modified QuEChERS methods for the analysis of fipronil and its metabolites in chicken meat

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Abstract. A sensitive method for the precisely and accurate determining the presents of fipronil and its metabolites in chicken meat was developed and validated using a modified quick, easy, cheap, effective, rugged and safe approach coupled with gas chromatography-mass spectrometry analysis. The solvent acetonitrile was used for the extraction of the samples with the salt phases composed of sodium chloride and magnesium sulphate, and then in the second phase used C18 and anhydrous magnesium sulphate. The linearity of the analytical response across the studied range of concentrations (0.005-0.050 mg kg⁻¹) was excellent, obtaining correlation coefficients higher than 0.99. The average recoveries of the pesticide ranged from 75 to 106% for fortification levels of 0.005, 0.01 and 0.05 mg kg⁻¹. The precision values associated with the analytical method, expressed as RSD values, were less than 11.15%. Matrix-matched solutions were also prepared by serially diluting the intermediate solution with blank chicken meat sample extracts containing none of the tested analytes to perform matrix-matched calibration with the same concentrations as in the solvent. The validated method was used to analyse the target compounds in 30 real samples imported from European countries. The present of fipronil-desulfanyl metabolite was confirmed in four samples.

1. Introduction

Pesticides are constantly used in agricultural and livestock production [1]. But pesticide residues can remain in the environment and pollute surface water, fruits and vegetables. When pesticides are used irresponsibly, disrespecting the waiting period between applications and maximum dosage levels, dangerously high residues of these products can be found in the foods produced from animals [2]. These compounds have massive potential dangers to human and environment health. The contamination routes can lead to bioaccumulation of persistent pesticides in food products of animal source such as meat, fat, fish, eggs and milk. The analysis of pesticide residues in food is very important due to the risks that these compounds offer to human health, besides their persistence in the environment and their ability to bioaccumulate [3]. Different pesticides can be imported into the food chain by usage of veterinary drugs and retained in the meat and meat products.

The situation that broke in Europe in July 2017, when millions of eggs were withdrawn from the shelves of stores across Western Europe due to high levels of the pesticide fipronil, has proved the need for improving and validating methods for accurate and precise analysis of fipronil in all matrices. Fipronil is a broad-spectrum insecticide that belongs to the phenyl pyrazole chemical family [4]. According to the IUPAC nomenclature, its name is (±)-5-amino-1-(2,6-dichloro-α,α,α, - trifluoro-p-tolyl)-4-trifluoromethylsulfinylpyrazole-3-carbonitrile. Fipronil was first registered for use by the United States Environmental Protection Agency (U.S. EPA) in May 1996. It is used as an active substance in veterinary products against fleas and ticks in dogs and cats. This formula is used in granular, gel and spray formulation for kills unwanted arthropods in horticultural, and also for kills ants and roaches. The two most represented metabolites of fipronil are fipronil sulfone and fipronil desulfanyl, of which fipronil sulfone is the most
widely used. A range of methods and techniques used to determine the levels of fipronil and these two metabolites has been addressed in a number of studies [5]. Also, a large number of studies examined the amount of fipronil in sediment and soil [3, 6], because they are the result of the integration of and chemical that take place in an aquatic ecosystem, affecting all processes in the whole system.

A number of analytical methods for extraction and analysis of multiple pesticide residues have been developed in the middle of the last century and have greatly contributed to agriculture, control and health care. During the 2000s, new advanced technologies incorporated the QuEChERS method that reduces the use of expensive and carcinogenic solvents in large quantities [7]. Different sorbents have different affinities for pesticide residues: for example, magnesium sulphate (MgSO₄) is used to reduce the water phase and promote partitioning of pesticides into an organic layer, while sodium chloride (NaCl) is used to dissolve fat globules. Primary secondary amine (PSA) is compound which can be used to remove substances such as fatty acids, organic acids and cholesterol. PSAs are especially useful for removing matrix co-extractants, which can interfere with pesticide determination. QuEChERS methods are increasingly being used because of the simple and quick preparation for a variety of matrices, such as soil, sediment, water [8] and various food matrices, among others, honey [9] and eggs [10]. Recently, the QuEChERS multi-residue procedure has replaced many complex and time-consuming analytical steps in traditional approaches and is being commonly used for the analysis of pesticides in foods. In many cases the main problem during analysis of pesticide residue is the purification process, which is required to isolate the residues from matrix components and reduce matrix effects and it is also essential to enable a long column life [11].

This study describes the use of a quick, easy, cheap, effective, rugged, and safe (QuEChERS) method for extraction and cleanup of fipronil and its metabolites of interest in chicken meat. Current developments involve the use of extraction methods based on modifications to the QuEChERS procedure. The aim of this study was to apply and validate methods for extraction and analysis of pesticide residues in chicken meat. The QuEChERS method was modified in the clean-up step by applying dispersive SPE using only the C18 and magnesium sulphate phase, which was recommended for the determination of drug residues in meat, versus standard dispersive SPEs that also contain PSA. The validation was performed by matrix-matched calibration, to compensate for the matrix effect. The quantification and identification of the pesticides were performed by gas chromatography combined with mass spectrometry (GC-MS). The prepared samples were analysed with GC-MS in the selected ion monitoring mode (SIM) using one target and three qualitative ions for each analyte. The method was validated using quality control material for fipronil in products of animal origin produced by Fapas (UK) in 2019.

2. Materials and methods

Quantification and quantification of analytes was carried out using a GC Clarus 680 PerkinElmer system comprising an autosampler and a gas chromatograph interfaced with an MS Clarus SQ8T instrument under the following conditions: capillary column Elite-5MS (30 x 0.25 mm ID x 0.25 µm df, composed of 95% dimethylpolysiloxane and 5% phenyl), operating in the electron impact mode at 70 eV. Helium (of the highest purity) was used as the carrier gas at the constant pressure of 21.5 psi and an injection volume of 2 µL was employed (a split ratio of 50:1) at the injector temperature of 250 °C; the temperature of the ion-source was 280 °C. Mass spectra were taken at 70 eV; the scan interval was 0.2 seconds and fragments were from 50 to 400 Daltons. The initial oven temperature of 70 °C was held for 3 minutes and then increased to 150 °C at 25 °C/min, then increased to 200 °C at 3 °C/min, and further increased to 280 °C at 8 °C/min and held for 10 min. Total GC running time was 41.87 min. The software was adapted to handle mass spectra and the chromatograms were read using Turbo Mass Ver 6.1.0.

Stock standard solution (10 mg L⁻¹) was supplied by DR Ehrenstorfer (LGC, Germany). A stock standard solution was prepared in acetonitrile and stored at -18 °C. The individual stock standard solutions were prepared in ethyl acetate. This solution was used as spike solution for recovery
experiments in the three-concentration level (0.005, 0.01 and 0.05 mg kg⁻¹) and also to prepare the analytical curves solution for linearity studies in solvent and in the matrix. All high purity solvents were obtained from PanReac AppliChem (ITW Reagents, USA). Extraction kits, apropos QuEChERS extraction agents, were manufactured by Phenomenex (USA). In order to investigate the matrix effect and to reduce matrix induced effects during GC, validation was performed by calibration in the matrix, so that the sample and calibration solution had the same concentration of co-extracted matrix components. Matrix-matched solutions were also prepared by serially diluting the intermediate solution with blank chicken meat sample extracts containing none of the tested analytes to perform matrix-matched calibration with the same concentrations as in the solvent. The use of matrix matched calibration solution is necessary to minimize errors associated with matrix induced enhancement or suppression effects during GC-determination. To each aliquot of the blank extract were added corresponding known amounts of pesticide standards mixture (five levels) and known and the same concentration of internal standard (ISTD). Triphenyl phosphate (TPP) was used as an internal standard.

Each sample was homogenized and ground using the laboratory blender and a representative amount of each homogenate (10.0 g) was then placed into a 50 mL polyethylene tube. Homogenates were extracted and cleaned up immediately after sampling using the QuEChERS method. Ten millilitres of acetonitrile were added into each tube. The samples were well shaken using a vortex mixer at maximum speed. Afterward, 4.0 g of anhydrous magnesium sulphate and 1.0 g of sodium chloride were added, then extracted by shaking vigorously on a vortex for 2 min and centrifuged for 5 min at 4000 rpm. An aliquot of 6 mL was transferred from the supernatant to a new clean 15 mL centrifuge tube containing C18 sorbent, and anhydrous MgSO₄ in a simple approach termed dispersive solid-phase extraction (dispersive-SPE) clean-up. The samples were again vortexed for 3 min and then centrifuged for 5 min at 4000 rpm. Final extracts of acetonitrile were concentrated using a gentle stream of nitrogen and reconstituted into ethyl acetate. To achieve the best possible extraction results, extracts were re-purified using the C18 column (100 mg/3 ml) before the analysis. The eluents were collected and were determined by precise GC/MS analysis using the selected ion monitoring mode with ion ratios for confirmation. After all determination parameters have been successfully set, the method was validated according to linearity, recovery, and precision.

3. Results and discussion

Trace analysis of organic contaminants, such as pesticides in food samples, typically consist of following consecutive steps: homogenization of samples, isolation of analytes from the sample matrix, removal of bulk co-extracts from crude extract, identification and quantification of target analytes and examination to ensure there have been no false positive results. Animal-derived food matrices are so chemically complex that sample preparation is extremely important for trace analysis, during which avoiding the interference caused by co-extraction of non-target substances is the biggest challenge and difficulty. Meat products contain a high content of lipids, i.e., more precisely myristic, palmitic and oleic acid, which can negatively affect the recovery of pesticides and damage the column and the baseline response. Modifications to the original QuEChERS method have been made depending on the characteristics of the samples, and in this case, because of the presence of lipids, so we opted for C18 purification without adding water, because we determined satisfactory recovery by optimization. For the method described here, the first phase without addition of water was found to be the most suitable.

Extraction is a highly significant part of sample preparation because it affects the efficiency of the purification process as well as the productivity of the clean-up step [10]. Therefore, due to the presence of proteins and fats, that is saturated and unsaturated fatty acids, additional purification was used using the C18 cartridge. This purification process has led to a higher sensitivity, less impact on the matrix effect, and a lesser presence and effect of fatty acids, esters and cholesterol during chromatogram recording in full scan mode [11]. Although a C18 column cartridge improved recovery of water-soluble pesticides, it was actually used in the last preparation step of the QuEChERS method.
in order to protect the GC column and MS detector. The QuEChERS method proved has been demonstrated to be an effective and versatile method of choice for extraction and has also been recently applied for the analysis of various xenobiotics and veterinary drugs in animal products [12].

The retention times of fipronil desulfinyl, fipronil and fipronil sulfone were 16.80, 21.25 and 24.45 min, respectively. The linearity of the analytical response across the studied range of concentrations (0.005-0.050 mg kg⁻¹) was excellent, obtaining correlation coefficients higher than 0.99 (Table 1).

| Analyte          | Regression equation         | R²       |
|------------------|-----------------------------|----------|
| Fipronil desulfinyl | y=0.227182x + 3.86920     | 0.999147 |
| Fipronil          | y=0.281971x – 10.2428      | 0.997444 |
| Fipronil sulfone  | y=0.245241x – 9.58740      | 0.999139 |

The validation parameters are based on the Document SANTE/12682/2019 [13]. The average recoveries of the residues ranged from 75 to 106%, for fortification levels of 0.005, 0.01 and 0.05 mg kg⁻¹ (Table 2). The precision values, expressed as RSD values, were less than 20% (Table 2). The LOQ is defined as the lowest validated spiked level (mean value for n=6) meeting the method performance acceptability criteria (mean recovery was in the range 70-120 %, with RSD ≤ 20%).

| Analyte          | Spiked (mg kg⁻¹) | Recovery (%) | RSD (%) | LOQ (mg kg⁻¹) |
|------------------|-----------------|--------------|---------|---------------|
| Fipronil desulfinyl | 0.005    | 85.63        | 11.15   | 0.003         |
|                  | 0.010    | 78.45        | 7.15    |               |
|                  | 0.050    | 86.36        | 3.45    |               |
| Fipronil          | 0.005    | 75.06        | 7.12    | 0.003         |
|                  | 0.010    | 94.56        | 6.13    |               |
|                  | 0.050    | 91.12        | 2.13    |               |
| Fipronil sulfone  | 0.005    | 103.52       | 6.94    | 0.003         |
|                  | 0.010    | 105.14       | 5.41    |               |
|                  | 0.050    | 106.09       | 3.94    |               |

Table 1. The linearity of the pesticide and residues at concentrations 0.005-0.05 mg kg⁻¹

Fipronil sulfone is the main metabolite of fipronil, deriving from the oxidation of the sulphynyl moiety. This metabolite showed an efficacy similar to fipronil against insect gamma-aminobutyric acid (GABA) receptors [14]. Practically, no clean-up method completely removes all the matrix components from a crude extract. Clean-up methods, where EMR-Lipid d-SPE extraction kits were used, gave the best reduction of co-extracted matrix compounds [15]. Figure 1 shows a chromatogram.
in full scan where the presence of niacinamide, one of two forms of vitamin B3, can be seen at a retention time of 7.14 min. Niacinamide is found in many foods including yeast, meat, fish, milk, eggs, green vegetables, beans and cereal grains. The following fatty acids are also present in the extract: 9-octadecenoic acid (Z) - methyl ester (oleic acid); 9,12-octadecadienoic acid (Z, Z) (linoleic acid) and 5,8,11,14-eicosatetraenoic acid, methyl ester (arachidonic acid methyl ester) at the retention time 26.60 min, 31.12 min and 32.72 min, respectively.

![Figure 1. GC-MS chromatogram of chicken meat sample in the full scan](image)

Development of certain methods for the detection of pesticides in food is fundamental for guaranteeing the nutritional quality and safety of food and the consumer health. In research, the results show the presence of fipronil in meat samples had a adverse effect on nutritional quality, causing a decrease in the amount of total and essential amino acids [16]. Using the QuEChERS method for the determination of fipronil and other pesticides, such as carbaryl, fenpyroximate, thiamethoxam, boscalid and difenoconazole, results were obtained that were in real samples at low concentrations and below acceptable limits [17]. During the extraction, water can also be used as an extraction agent, whereby no satisfactory recovery was obtained for the determination of fipronil in melon, while a satisfactory recovery was obtained for the determination of fipronil in milk [18]. On the other hand, successful validation of fipronil by the QuEChERS method was achieved in beef meat using liquid chromatography coupled to mass spectrometry detection (LC-MS), while in the examined real samples, the amounts of pesticides were the result of LOQ [19]. Animal products are matrices characterized by high water, protein and lipid concentration that require the use of complex methods for cleanup and extraction. In published papers on the presence of pesticides in meat, quantified amounts in meat were determined for the pesticides carbaryl and chlorpyriphos ethyl [20].

The maximum residue limit (MRL) set for fipronil in eggs and chicken meat is 0.005 mg kg\(^{-1}\) (EC No 396/2005). This MRL value has been set for the sum of fipronil and its sulfone metabolite (expressed as fipronil). Fipronil used to prepare the formulations can contain fipronil sulfone at the time of formulation. In other words, it is possible that useful grades of fipronil have some amount of fipronil sulfone already present as a by-product of its preparation. Some grades of fipronil can have low levels of fipronil sulfone, even 0%. However, once fipronil has contacted oxidation agents, it is believed that fipronil sulfone could continue to form in solution. Further, it is significantly more costly to use fipronil that is completely free of fipronil sulfone. Using this validated method, the accuracy of the method was confirmed using the reference meat material from Fapas (13.9 ± 3.5 mg kg\(^{-1}\)).
In the tested real samples of chicken meat, the amount of fipronil and its metabolites was below the LOQ. The metabolite fipronil-desulfinyl was detected in four of the samples of chicken meat. The results of the analysis of fipronil-desulfinyl in the tested samples ranged from 0.004 to 0.006 mg kg$^{-1}$.

4. Conclusion
Successfully developed new method of analysis proved to be able to meet the requirements of modern methods in the field of quantification of fipronil in food of animal origin, i.e. meat. The validation data of chicken meat sample proved the compliance of the new analysis method with the legal requirements of the quality control criteria according to the SANTE 12682/2019 document. This confirmed that the results achieved by means of the newly developed analysis method are in accordance with the currently available sample preparation methods. Consequently, the presented analysis method is suitable for application for other matrices in routine analysis. Successful application of this method is relevant to monitoring the impact of fipronil contamination in the environment, primarily through monitoring the presence of fipronil in food, which will provide better insights into the present situation of the environment. Consequently, the successful investigation of the quality test materials proved the applicability of the presented analysis method as a confirmation method in routine analysis, quantifying the same amounts as were declared. Therefore, it is expected that new modifications to the QuEChERS methodology will continue to be developed to expedite the analysis of more veterinary drugs and other xenobiotics in animal products, and the methodology could become a basic choice for monitoring most contaminants in the future.

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