

Determination of pesticide residues in plant-based foods from the Republic of Macedonia

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Abstract: The present study investigates pesticide residues in samples of fresh and processed fruits and vegetables produced in Republic of Macedonia. Investigations were carried out as a part of the National Monitoring Programme under the recommendations of the European Food Safety Authority (EFSA). From September 2012 to June 2013, a total of 168 samples of different fresh vegetables: tomato, paprika, cucumber, potato, onion, carrot, cabbage; processed vegetables: pasteurized paprika, ketchup; fresh fruits: apple, acid cherry, table grapes and wine grapes; processed fruits: jams and canned fruits were tested on the presence of 33 pesticide residues. The QuEChERS procedure was used for sample preparation, except for dithiocarbamates and gas chromatography – mass spectrometry (GC-MS) and liquid chromatography – tandem mass spectrometry (LC-MS/MS) techniques were applied for pesticide residues identification and determination. The results of the study showed that cucumber was the crop with the highest number of pesticide residues with the predominant presence of methomyl, (0.015-0.21 mg/kg), metalaxyl (0.04-0.16 mg/kg), and imidacloprid (0.017-0.036 mg/kg). Only two samples (1.19% of the total samples) contained one pesticide residue above the Maximum Residues Levels (MRLs). The results of the current study showed that 98.8% of the tested samples contained residues below the MRLs.

Keywords: Pesticide Residues, Fungicides, Insecticides, GC-MS, LC-MS/MS

1. Introduction

Most fruits and vegetables are treated with pesticides on several occasions during the growing season. Pesticides enable farmers to produce some fruits and vegetables in areas that otherwise would not be suitable, increase their yields, preserve product quality, and extend shelf life. Without pesticides, commercial fruits and vegetables production would not be economically viable in many regions of the world. Insecticides and fungicides are likely to remain the major class of pesticides used for crops protection in developing countries. Insecticides are used to control pests [1]. Fungicides are applied to control a considerable number of diseases caused by *Venturia inaequalis*, *Uncinula necator*, *Sclerotinia blight*, *Botrytis cinerea* etc [2]. At the same time, pesticides can pose risks if they are not applied according to Good Agricultural Practice (GAP). Pesticide levels tend to decline over time as the

residues on crops degrade/metabolise during their growing period and following harvest if they are washed and processed before reaching the markets. Many authors have also shown that if pesticides are applied to grapes in accordance with GAP then the Maximum Residue Levels (MRLs) will not be exceeded [3, 4]. So, levels are usually well below legal limits by the time food reaches the retail outlets.

Pesticides are among the most regulated products in the world. Because of their importance in terms of consumer safety, pesticide residues have been determined using methods based on gas chromatography – mass spectrometry (GC-MS) [5-9, 12, 14-15], liquid chromatography-tandem mass spectrometry (LC-MS/MS) [5-6, 10-12, 13, 15], liquid chromatography with fluorescence detection (LC-FLD) [7] or liquid chromatography with UV detection (LC-UV) [8].

The Republic of Macedonia is an agricultural developing country with a large production of different types of vegetables and fruits in open fields as well in greenhouses. In order to apply a National Monitoring Programme for pesticide residues control according to the recommendation of European Food Safety Authority (EFSA), the Macedonian Food Agency conducted a study on the determination of pesticide residues. The aim of this study is to investigate the occurrence and concentrations of pesticide residues in some fresh and processed vegetables and fruits from domestic producers.

2. Materials and Methods

2.1. Sampling

The sampling was focused at the markets, as the end point of the food production chain. From September 2012 to June 2013, a total of 168 samples of different fresh vegetables and fruits (tomato, paprika, cucumber, potato, onion, carrot and cabbage, apple, acid cherry, table grapes and wine grapes) and processed vegetables and fruits (pasteurized paprika, ketchup, jams and canned fruits) were tested to detect the presence of residues of the 33 pesticides above indicated. Samples were taken from markets located in the regions of the country. All samples were produced in the Republic of Macedonia. The sampling scheme was based on a combination of random and target oriented samples. The target oriented sampling was planned based on: dietary consumption of the food product in question, amount of the production/distribution, seasonal particularities, and the chemical class of pesticides used. Blank samples of cabbage, tomatoes and grapes were purchased from a local organic producer. These blank samples were used for preparation of matrix blanks and matrix-matched calibration standards.

2.2. Reagents

The different chemical classes and pesticides included in this study were: phthalimide fungicides (folpet, captan); triazole fungicides (tebuconazole, propiconazole); anilide fungicides (boscalid, fenhexamid, metalaxyl); anilinoimidazole fungicides (cyprodinil); dithiocarbamate fungicides (mankozeb, propineb); neonicotinoid insecticides (acetamiprid, imidacloprid); triazine acaricides/insecticides (cyromazine); pyridine azomethine insecticides (pymetrozine); carbamate acaricides/insecticides (methomyl); organothiophosphate insecticides/nematocides (terbufos); organothiophosphate acaricides/insecticides/nematocides (chlorpyrifos, dimethoate); organothiophosphate acaricides/insecticides (azinphos-methyl, diazinon, malathion, phosmet, pirimiphos-methyl); organothiophosphate insecticides (fenitrothion, methidathion); organophosphate acaricides/insecticides (dichlorvos monocrotophos); synthetic pyrethroid insecticides (deltamethrin); synthetic pyrethroid acaricides/insecticides (bifenthrin, lambda cyhalothrin, alpha cypermethrin); and the metabolites of organochlorine

insecticides (endosulfan sulfat ; o,p'-DDD).

Reference pure standards of the pesticides analyzed and carbon disulphide (CS₂) were obtained from Dr. Ehrenstorfer (Augsburg, Germany) and Sigma-Aldrich/Fluka/Riedel-de-Haen (Zwijndrecht, The Netherlands). Triphenylphosphate (TPP) and ethoprophos were obtained from Merck (Darmstadt, Germany). Stock solutions of 1.00 mg/mL were prepared in toluene and methanol for the pesticides analyzed by GC-MS and LC-MS/MS, respectively. For captan and folpet, individual working standard solutions of 5 ng/μL and 0.5 ng/μL were prepared in acetonitrile. Working standard mixtures, containing the 21 pesticides analyzed by the GC-MS multiresidue method or the 8 pesticides analyzed by the LC-MS/MS multiresidue method, were prepared in acetonitrile at concentrations of 5 ng/μL and 0.5 ng/μL for each pesticide. A solution of 2 ng/μL TPP was prepared in acetonitrile with 2% acetic acid (v/v) (to be used as the QC standard for the GC-MS determination). A 5 ng/μL ethoprophos solution was prepared in acetonitrile (to be added to the samples as the IS for GC-MS and LC-MS/MS determinations).

For analysis of dithiocarbamates, a stock standard solution of carbon disulphide (CS₂) in isooctane (residues analysis grade) with approximate concentration of 630 μg CS₂/mL was prepared as follows: 50 μL of CS₂ were added to a 100 mL volumetric flask containing approximately 95 mL of isooctane. Because of its volatility, carbon disulphide was added using a syringe under the surface of the solvent. The flask was weighed before and after the addition of carbon disulphide and then filled to the designated mark with isooctane. At the end the actual concentration of the solution was calculated. An intermediate solution with the concentration of approximately 6.3 μg CS₂/mL, which is equivalent to 5.0 mg CS₂/kg of sample, was prepared from this solution. Working solutions with the concentrations: 0.05, 0.06, 0.25, 0.63, 1.26 and 3.15 μg CS₂/mL were prepared from the intermediate solution, which is equivalent to 0.04, 0.05, 0.20, 0.50, 1.01 and 2.52 mg CS₂/kg of the sample. For captan and folpet, stock solutions of 1.00 mg/mL were prepared in toluene and methanol. Working standard solutions of 5 ng/μL and 0.5 ng/μL were prepared in acetonitrile. Deuterated internal standards solutions of captan D6 (100 ng/μL) and folpet D4 (100 ng/μL) were obtained from Dr. Ehrenstorfer.

2.3. Analytical Methods

2.3.1. Multi-Residue Method

In the current study we used the QuEChERS extraction procedure for sample preparation. An aliquot of 15 g of homogenized sample was placed in a 50 mL centrifuge tube and 15 mL 1% acetic acid in acetonitrile (v/v) was added together with 6 g magnesium sulfate and 1.5 g sodium acetate. The tube was shaken and centrifuged. A portion of the extract was mixed with 3 + 1(w/w) mixture of magnesium sulfate and primary secondary amine sorbent (200 mg/mL extract) and centrifuged once again. The

aliquots of the extract (1 mL) were transferred to separate autosampler vials for GC-MS and LC-MS/MS determination.

For preparation of the matrix-matched calibration standards, cabbage, tomato and grapes were used as representative matrices. In each case, 6 replicates each at 4 levels (10, 25, 50, and 100 ng/g) were fortified into the samples. An aliquot of 15 g of homogenized sample was placed in a 50 mL centrifuge tube (15 mL deionized water were used for a reagent blank). A 15 g blank was prepared as the matrix blank and for matrix-matched calibration standards. The samples were fortified with the 300 μ L of the 0.5 ng/ μ L spiking mixtures and 75, 150, and 300 μ L of the 5 ng/ μ L spiking mixtures, to yield 10, 25, 50, and 100 ng/g concentrations, respectively. 15 mL 1% acetic acid in acetonitrile (v/v) was added together with 6 g magnesium sulfate and 1.5 g sodium acetate. 300 μ L of the 5 ng/ μ L ethoprophos solution in acetonitrile was added to all samples except blanks (this gives a 100 ng/g equivalent concentration). The tube was shaken and centrifuged. A portion of the extract was mixed with 3 + 1(w/w) mixture of magnesium sulfate and primary secondary amine sorbent (200 mg/mL extract) and centrifuged once again. The aliquots of the extract (1 mL) were transferred to separate autosampler vials for GC-MS [12] and LC-MS/MS [13] determination.

GC-MS determination: 50 μ L TPP (2 ng/ μ L) in acetonitrile containing 2% acetic acid (v/v) was added to each GC-MS vials containing sample and blank extracts. Analysis was performed using a GCMS-QP2010 chromatograph (Shimadzu) instrument. An Rtx-5MS capillary column (30 m x 0.25 mm ID x 0.25 μ m film thickness) was used to separate the pesticides. Sample injection was performed in the splitless mode with a sampling time of 1 min (high pressure injection 250 kPa 1.5 min). The injector temperature was 250 $^{\circ}$ C, the ion source temperature was 230 $^{\circ}$ C, the auxiliary temperature was 280 $^{\circ}$ C, and quadrupole temperature was 150 $^{\circ}$ C. The GC oven temperature was programmed from 50 $^{\circ}$ C (held for 1 min) to 125 $^{\circ}$ C at rate 25 $^{\circ}$ C/min (held for 2 min), then to 180 $^{\circ}$ C at rate 5 $^{\circ}$ C/min (held for 30 min), then to 230 $^{\circ}$ C/min at rate 20 $^{\circ}$ C/min (held for 15 minutes), then to 250 $^{\circ}$ C at rate 20 $^{\circ}$ C (held for 12 min), then to 300 $^{\circ}$ C at rate 20 $^{\circ}$ C/min (held for 15 min). The carrier gas (helium) constant flow was 4.2 cm/sec. The following 21 pesticides were analyzed using GC-MS: azinphos-methyl; bifenthrin; boscalid; chlorpyrifos; lambda cyhalothrin; alpha cypermethrin; cyprodinil; o,p'-DDD; deltamethrin, diazinon; dichlorvos; dimethoate; endosulfan sulfat; fenitrothion; malathion; metalaxyl; monocrotophos; pirimiphos-methyl; phosmet; tebuconazole and terbufos.

LC-MS/MS determination: LC-MS/MS determination was undertaken using a rapid resolution LC (Agilent 1200) system coupled with a triple quadrupole mass detector (Agilent 6460). The mass spectrometer was operated in the electrospray ionization (ESI) mode. The electrospray temperature was kept at 350 $^{\circ}$ C and the sheath gas flow was

11 L/min. Pesticides were separated on a Zorbax C18 column, 10 mm x 2.1 mm with 1.8 μ m particle size (Agilent). Gradient elution was used for pesticide separation. Mobile phase A was 10 mM ammonium acetate in water. Mobile phase B was methanol. The injection volume was 10 μ L. The flow rate of column was set at 0.4 mL/min. The oven temperature was 40 $^{\circ}$ C. The initial conditions (100% mobile phase A) were maintained for 2 min, then a linear gradient was applied (5-98% mobile phase B) over 12 min and to 100% mobile phase B in 15 min. Conditioning of the column with the initial mobile phase A was carried out for 10 min. Data collection was performed in multi reaction monitoring mode (MRM) with a dwell time of 1 ms. For each compound, two MRM transitions were monitored. The following 8 pesticides were determined using LC-MS/MS: acetamiprid, cyromazine, fenhexamid imidacloprid, methidathion, methomyl, propiconazole and pymetrozine.

2.3.2. Method for Dithiocarbamates

50 g of the sample was placed in a laboratory bottle where 40 mL isoctane was added with a pipette and a 100 mL solution of stannous (II) chloride (2 % w/v) was added using a graduated cylinder. The closed bottle was placed in a water bath at 80 $^{\circ}$ C and heated for 60 minutes. The solution was mixed approximately every 15 minutes. The bottle was taken out of the bath after one hour, cooled to room temperature and the upper phase was transferred into a GC vial using a Pasteur pipette. Spiked samples were treated in the same way. For each series of analysis a reagent blank was prepared. [14] The GC-MS system for the determination of carbon disulfide was the same as that described for multi-residual GC-MS determination, but operating using a different temperature programme. An Rtx-5MS capillary column (30 m x 0.25 mm ID x 0.25 μ m film thickness) was used to separate the pesticides. Sample injection was performed in the splitless mode (injection volume 1 μ L). The injector temperature was 250 $^{\circ}$ C, the ion source temperature was 230 $^{\circ}$ C, the auxiliary temperature was 280 $^{\circ}$ C, and the quadrupole temperature was 150 $^{\circ}$ C. The GC oven temperature was programmed from 50 $^{\circ}$ C (2 min) to 270 $^{\circ}$ C at rate 35 $^{\circ}$ C/min (held for 5 min).

2.3.3. Single – Residue Method for Captan and Folpet

We used the same QuEChERS extraction procedure for sample preparation as for the multiresidue method. The corresponding deuterated internal standards captan D6 and folpet D4 (100 μ L of a 10 μ g/mL in acetonitrile) were added in the centrifuge tube prior the extraction. 30 μ L of shikimic acid (5 mg/mL) in acetonitrile: water (7:3 v/v) was added per 1 mL of the final extract prior to GC-MS analysis. Spiked samples were treated in the same way (matrix blank plus 3 spiked concentration levels of 10, 25, and 50 ng/g). For each series of analysis a reagent blank was prepared. Determination of captan and folpet residues was made by gas chromatography/negative chemical ionization mass spectrometry [15]. An Rtx-5MS (30 m x 0.25 mm ID x 0.25 μ m film thickness) was used to separate the pesticides. The GC oven temperature was programmed from 40 $^{\circ}$ C (held for

2 min) to 220 °C at rate 30 °C/min, then to 260 °C at rate 5 °C/min, and then to 280 °C/min at rate 20 °C/min (held for 15 minutes). The analyte concentration was calculated via regression curve of the area ratios of the analyte to the corresponding internal standard.

3. Results and Discussion

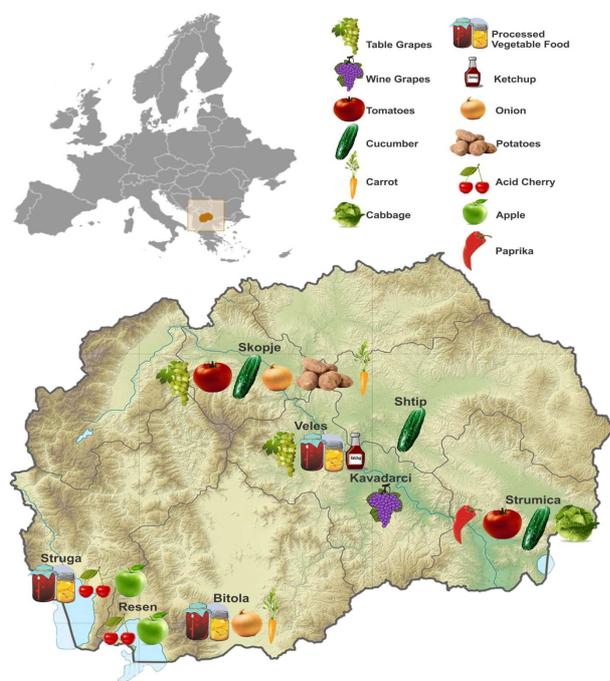


Figure 1. Regions of food sampling

The national pesticide monitoring was performed according to a nation-wide sampling plan designed by the Macedonian Food Agency. The plan was based on data concerning dietary consumption and production of fruits and

vegetables. Furthermore the residue results of earlier monitoring - programmes and the data on the usage of pesticides on vegetables and fruits protection in the country were also taken into account. In addition routine samples were taken from the Macedonian market by responsible staff. All samples were taken from the markets from several different regions in the country (Fig. 1).

From September 2012 to June 2013, a total of 168 samples of different fresh and processed vegetables and fruits were tested on the presence of 33 pesticide residues under the national pesticide monitoring programme as routine samples. Fig. 2 shows the percentage (%) of tested samples.

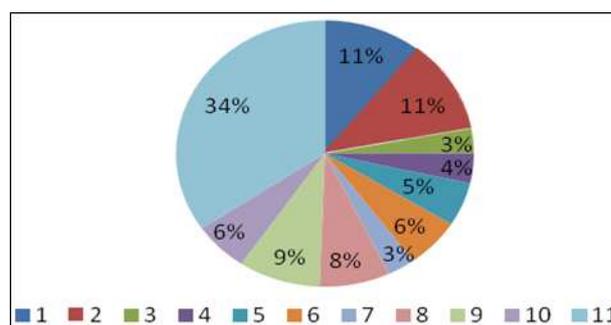


Figure 2. The percentage (%) of each food commodity (total of 168) included in the 2012-2013 study (1-cucumbers; 2-grapes; 3-tomatoes; 4-carrots; 5-potatoes; 6-acid cherries; 7-apples; 8-onion; 9-paprika; 10-cabbage; 11-processed vegetables and fruits)

The results of our investigation showed that cucumbers were the vegetables which had the highest abundance of pesticides. Pesticides residues detected in cucumbers are shown in Table 1.

Table 1. Pesticides residues found in cucumbers

Pesticide	Number of positive samples (n)	Range (mg/kg)	Mean \pm SD (mg/kg)	MRL (mg/kg)
Boscalid	3	0.04-0.09	0.067 \pm 0.025	3
Chlorpyrifos	3	0.021-0.04	0.030 \pm 0.01	0.05
Cyprodinil	3	0.05-0.32	0.168 \pm 0.138	0.5
Fenhexamid	3	0.035-0.09	0.059 \pm 0.028	1.0
Imidacloprid	6	0.017-0.036	0.024 \pm 0.008	1.0
Metalaxyl and Metalaxyl M (sum of isomers)	6	0.04-0.16	0.073 \pm 0.044	0.5
Methomyl and Thiodicarb (sum of isomers expressed as Methomyl)	7	0.015-0.21	0.058 \pm 0.049	0.02
Tebuconazole	3	0.023-0.05	0.034 \pm 0.014	0.5

Among the most common pests attacking cucumbers are leaf mites (*Aphis craccivora*) and leaf beetles (*Aulacaphora spp.*). Common diseases are anthracnose (*Colletotrichum lindemuthianum*), downy mildew (*Pseudoperonospora cubensis*), rust mildew (*Erysiphe cichoracearum*), grey mould (*Botrytis cinerea*), which are controlled by farm sanitation and the usage of suitable pesticides (cyprodinil, fenhexamid, procymidone, metalaxyl etc.), according to the GAP [16]. The most abundant pesticide residues found in

cucumber samples were methomyl, metalaxyl and imidacloprid. Methomyl was present in 6 of tested samples (33.3 % of the total cucumber samples) at concentrations close to the MRL. One sample exceeded the MRL value by a factor of 10. The possible reason for this non-compliance is over application. In a study conducted by Mohamed and Saad [17] residues of methomyl were 0.6 mg/kg seven days after methomyl had been applied to cucumbers. Methomyl is a carbamate insecticide with restricted use because of its

high toxicity to humans. Therefore, the MRL for methomyl and thiodicarb (sum of methomyl and thiodicarb expressed as methomyl) for the most of fruits and vegetables is set at the 0.02 mg/kg in the Regulation (EC) 396/2005 [18]. According to the EFSA report on pesticides residues [19] in 2010, 6778 food samples were tested for methomyl and thiodicarb. 99.94% of tested samples did not contain measurable residues of methomyl.

Metalaxyl is a systemic phenylamide fungicide. The concentrations of metalaxyl expressed as metalaxyl and metalaxyl M (sum of isomers) in all positive samples were below the MRL (0.5 mg/kg).

Imidacloprid is a systemic insecticide that belongs to neonicotinoids and is currently the most widely used insecticide in the world [20]. Despite its widespread use, all positive samples had residues of imidacloprid well below the MRL (1 mg/kg). Low residues levels of imidacloprid

were reported in EFSA report [19], where only 3.44% of all the tested food samples (8082) contained residues below or at the MRL. No samples with residues that exceeded the MRL were found.

Chlorpyrifos is organophosphate insecticide that is acutely toxic to humans. 3 samples of cucumber contained chlorpyrifos within the range 0.021-0.04 mg/kg, which were all below the MRL (0.05 mg/kg). A residue of chlorpyrifos was found to exceed the MRL (0.1 mg/kg) in 1 sample of carrot (0.16 mg/kg). Residues of chlorpyrifos were also found in 1 sample of tomato (0.19 mg/kg) and in 3 apple samples (0.02-0.08 mg/kg), but they were below the MRL for both crops (0.5 mg/kg). The results of current study for pesticide residues in table and wine grapes showed that in 4 of the tested samples residues of boscalid were found within the range 0.06-0.12 mg/kg (Table 2).

Table 2. Pesticides residues found in grapes

Pesticide	Number of positive samples (n)	Range (mg/kg)	Mean \pm SD (mg/kg)	MRL (mg/kg)
Boscalid	4	0.06-0.12	0.092 \pm 0.027	5
Cyprodinil	3	0.014-0.018	0.017 \pm 0.003	5
Metalaxyl and Metalaxyl M (sum of isomers)	3	0.067-0.010	0.082 \pm 0.017	2

Cyprodinil (0.014-0.018 mg/kg) and metalaxyl (0.067-0.10 mg/kg) were found in three samples of table grapes. In both cases the residues were well below MRL (5mg/kg and 2 mg/kg, respectively). Interestingly, no residues of these 2 fungicides were detected in any of the samples of wine grapes. This was probably due to differences in climatic conditions between the two areas where the grapes were grown. In the cooler continental region of Macedonia, where the table grapes were grown, a more effective protection strategy against grapevine diseases is needed and consequently some fungicide residues were detected.

Crops with no detectable pesticide residues were: onion, potato, paprika, cabbage and acid cherry. In our study, 58 samples of processed vegetables: fried and pasteurized paprika (25), ketchup (12); processed fruits: processed fruits: plum jams (11) and canned apricots (10), were tested on the presence of 33 pesticide residues. No pesticide residues were detected in any of the samples of processed vegetables and fruits. This could be due to the pesticide degradation during the processing of the crops. According to Celik [21] *et al.*, the effects of the food processing techniques reduce the pesticide residue levels, except in cases where there is concentration of the product like in juicing, frying and oil production.

The results of the investigations showed that in 79.76% of the surveillance samples (vegetables, fruits and processed vegetables and fruits) no pesticide residues could be detected. 20.24% of the samples had residues under or at the (MRL) [18]. Only 2 samples (1.19% of the total samples) contained one pesticide above the MRL (methomyl in cucumber, and chlorpyrifos in carrot) and were therefore considered to be not-compliant.

4. Conclusion

The results of the current study showed that the 98.8% of the samples that were found to contain residues well below the relevant MRLs. Our investigations showed that cucumber contained the highest number of pesticides residues with a predominance of methomyl. The other pesticide residues found in cucumber samples were boscalid, chlorpyrifos, cyprodinil, fenhexamid, imidacloprid, metalaxyl and tebuconazole. Table grapes showed a predominance of boscalid residues, but all were below the MRL. Only two samples (1.19% of the total samples) contained one pesticide residue above the MRL (methomyl in cucumber, and chlorpyrifos in carrot). No pesticide residues were detected in any of the samples of processed vegetables and fruits.

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