ORIGINAL PAPER

DETECTION OF FUNGICIDE RESIDUES IN SOME FRUITS AND VEGETABLES BY CHROMATOGRAPHIC METHODS

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Abstract. *The use of pesticides is a common practice in modern agriculture. To increase and to ensure the security in people nutrition, two comparative chromatographic methods (GC-MS and HPLC-DAD) for fungicide residues detection and monitoring in some fruits and vegetables were tested. Some vegetables, eggplants, cucumbers, red potatoes, white potatoes, red peppers and fruits, plums, apples, lemons, grapes, clementines, were analyzed for fungicide residues quantification. The presence of Tebuconazole, Boscalid, Iprodione and Imazalil was followed and quantified in the tested samples. These analyses are important for environment and end-consumers protection. Generaly, both techniques are very sensitive and selective for the analysis of pesticides at low concentrations. GC-MS presents better linearity characteristics for Tebuconazole and Imazalil, but HPLC-DAD was the most rapid method. The tested products do not contain large fungicides quantities.*

Keywords: *fungicide quantification; ultrasound extraction; GC-MS; HPLC-DAD.*

1. INTRODUCTION

The pesticides thought to be the likeliest to cause some types of cancer [1]. Productivity of fruits and vegetables has been greatly enhanced by developing working techniques, the use of the best seeds, better water management, and also by the efficiency administration of pesticides. The increasing application of these compounds in various agricultural activities was accurately determined by monitoring of concentration levels for the protection of ecological systems and food supplies [2].

Fungicides are pesticides that can act as inhibitors of electron transport chain, inhibitors of enzymes, inhibitors of nucleic acid metabolism and protein synthesis and inhibitors of sterol synthesis. Fungicidal action can be expressed in one of two physically visible ways: inhibition of spore germination and inhibition of fungi growth [3].

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Boscalid, a pyridinecarboxamide, is a broad-spectrum fungicide from the succinate dehydrogenase inhibitor (SDHI) class [4] and is highly effective against a wide range of plant pathogens, *i.e., Sclerotinia sclerotiorum* [5], *Penicillium digitatum* [6], *Botrytis cinerea* [7], *Alternaria alternata* [8], etc. Imazalil, is a pesticide used as a broad-spectrum systemic imidazole fungicide, being the most widely used postharvest fungicide in citrus fruits for the control of green and blue moulds caused by *Penicillium digitatutm* and *P. italicum* [9, 10]. Tebuconazole, a triazole fungicide, acts as an effective multifunctional systemic fungicide by suppressing the ergosterol biosynthesis, preventing the formation of cell membranes, causing the death of pathogens [11, 12]. It is used as a seed dressing chemical and as foliar spray to protect cereal crops from different fungal diseases such as rusts, smuts, bunt, powdery mildew, leaf spots, and blight [13]. Iprodione, a contact dicarboximide fungicide acts as glycerol synthesis and hyphal development inhibitor. It is commonly used in vegetables, vines, and flower crops, to control *Botrytis cinerea* [14, 15].

Fruits and vegetables have a significant role in human nutrition and health [16, 17]. The use of pesticides to control fruits and vegetables disease can result in the presence of pesticide residues. The level of these residues can be below the maximum residue limit (MRL) if good agricultural practices are used.

According to Article 12 of Regulation (EC) No 396/2005, the European Food Safety Authority (EFSA) has reviewed the Maximum Residue Levels (MRLs) currently established at European level for the pesticide active substance including Boscalid, Tebuconazole, Iprodione and Imazalil [18-22].

To assess the occurrence of fungicide residues in plants, processed commodities, rotational crops, and livestock, EFSA considered the conclusions derived in the framework of Directive 91/414/EEC, the MRLs established by the Codex Alimentarius Commission, as well as the important tolerances and European authorizations reported by Member States (including the supporting residues data).

The dwindling MRLs have forced the development of fast and easy multi-residue methods using different extraction solvents and measurement procedures to ensure more sensitive analytical methods to meet the requirements in complex samples, like food [23, 24]. There are many possibilities to detect the pesticide residues in vegetables and fruits and it can be mentioned chromatographic techniques such as thin layer chromathography (TLC) [25], gas chromatography–mass spectrometry (GC-MS), liquid chromatography-tandem mass spectrometry (LC-MS/MS), high-performance liquid chromatography with diode-array detection (HPLC-DAD), ultra-high performance liquid chromatography combined with tandem mass spectrometry (UHPLC-MS/MS) [26-29], special affinity sensors (affinity sensors are based on the selective recognition of the biological or biomimetic analyte elements such as antigen-antibody binding, nucleic acid hybridization or synthetic receptor/target recognition: molecular imprinted polymers, aptamers, etc.) [30] and electrochemical methods [31, 32].

The aim of this work was to detect and quantify the residues of four fungicides: Boscalid, Tebuconazole, Iprodione and Imazalil in some vegetables and fruits using both liquid and gas chromatography (*i.e.*, HPLC-DAD and GC-MS). The chromatographic methods though involve the professional operation, periodical verification of apparatus, large quantities of reagents (or gas) and the transport of the samples in a lab, are sensitive, accurate, robust, and reliable.

2. MATERIALS AND METHODS

2.1. MATERIALS

The fungicides: Boscalid, Tebuconazole, Iprodione and Imazalil (Pestanal standards), acetonitrile (ACN), phosphoric acid and HPLC grade water were purchased from Sigma-Aldrich. The vegetables and fruits were achieved from supermarkets and local farmers.

2.2. METHODS

2.2.1. Extraction and samples preparation

The skin of vegetables and fruits were cutted in small pieces and were dried for complete water elimination. The extraction was carried out using ultrasounds, in one step, with acetonitrile, at 59 kHz, 30±2 °C, during 30 min. The ratio solvent: solid was 2-4:1 (v/w). The excess of solvent, after quantitative fast filtration on special paper, was evaporated in a rotary evaporator Laborota 4000 Efficient-Heidolph, and brought to 10 mL with acetonitrile. Before chromatographic analyses, the solutions were filtered through 0.2 µm PTFE filters.

2.2.2. GC-MS analysis

The GC-MS system Agilent 7890A-5975C VL-MSD equipped with Zebron ZB-35 (30-meter x 0.25 mm x 0.25µm) column was used. Helium was used as carrier gas at a constant flow 1 mL/min. The column temperature was set at 70 °C, held for 2 min., then programmed at 15 °C/min ramp rate to 200 °C, held for 1 min., then programmed at 40 °C/min ramp rate to 250°C, and held for 30 min. Injected volume was 0.2 µL at 260 °C, in split mode (split ratio 20:1). Time of analysis was about 40 min. Data acquisition and processing was performed with ChemStation G1701EA software.

2.2.3. HPLC-DAD analysis

A Dionex Ultimate 3000 (Dionex Corp., USA) equipped with a PDA 3000 photodiode array detector and a C-18 Acclaim® 120 Silica-Based reversed-phase (4.6 x 150 mm, 5µm) was used. Samples were analyzed at 30 °C, flow rate 0.5 mL/min. The concentration values for Boscalid, Iprodione and Imazalil were registered at λ=210 nm and for Tebuconazole at λ=220 nm. A mixture (70%: 30%) of acetonitrile and acidulated water (0.1% vol: vol, H_3PO_4 ≥85%) was used as mobile phase under isocratic conditions. The volume of each injection was 20 µL. Time of analyses was 15 min., except Imazalil (20 min.). Detection of Imazalil was carried out using the following method: isocratic conditions for 3 min. with 30% ACN and 70% acidulated water, linear gradient from 30% to 70% ACN between min. 3-10, and after min. 10, isocratic conditions until the end of analysis. Chromatograms were recorded and processed with Chromeleon 6.8 Software.

2.2.4. Determination of LOD and LOQ

There are many methods for detection limits of the low concentration, LOD and LOQ [33-35]. The one based on standard deviation of the response and the slope of a regression curve was chosen (RMSE). Samples were prepared in the range of LOD and LOQ; six or more determinations were made at five concentrations. The detection limits may by expressed as (Equations 1 and 2):

$$LOD = 3.3 \cdot \sigma / S \quad (1)$$

$$LOQ = 10 \cdot \sigma / S \quad (2)$$

where σ is the standard deviation of the response at low concentrations and S is the slope of the calibration curve.

The slope was estimated from the calibration curve of each analyte. The estimate of σ is the root mean squared error (RMSE) or standard deviation of residuals taken from the regression line (Equation 3).

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n}(Pi-Oi)^2}{n}} \quad (3)$$

where: *Pi* = predicted values
Oi = observed values
n = number of samples.
The smaller RMSE value, the closer predicted and observed values.

2.2.5. Determination of precision

For judging the precision of the method, the concentrations of 0.19, 0.38, 0.96, 1.92, 3.83 and 7.66 µg/mL of Iprodione were used for HPLC analyses. The eggplant samples were contaminated with Iprodione in acetonitrile before extraction. After extraction, five injections for each concentration in the same day were carried out, the same was done for the next two days in order to assess the intra-day and inter-day reproducibility of results.

2.2.6. Statistical analysis

Statistical analysis was performed using OriginPro 6 software. Data regarding fungicide standards and fungicide presence in samples were expressed as mean ± standard deviation (SD).

3. RESULTS AND DISCUSSION

Some vegetables and fruits, both local and imported (Table 5), were submitted to ultrasonication in acetonitrile, the resulted extracts were then analyzed for fungicide residues quantification, using GC-MS and HPLC-DAD techniques. The presence of Tebuconazole, Boscalid, Iprodione and Imazalil was evaluated in the studied samples.

In Figs 1-4, the chromatographic profiles of Imazalil and Iprodione are shown. Calibration curves for each type of fungicide using GC-MS and HPLC-DAD methods have been plotted (Figs. 1, 2, and 5). Each point was obtained as the average value of six injections.

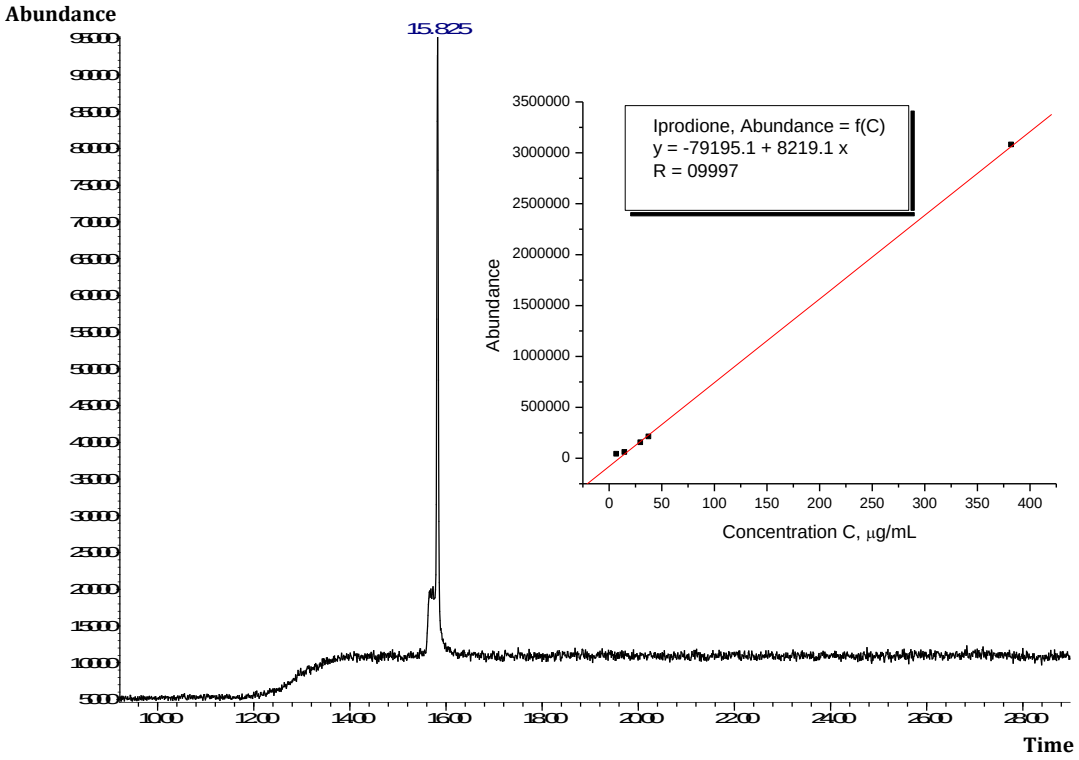

Figure 1. GC chromatogram and calibration curve of Iprodione.

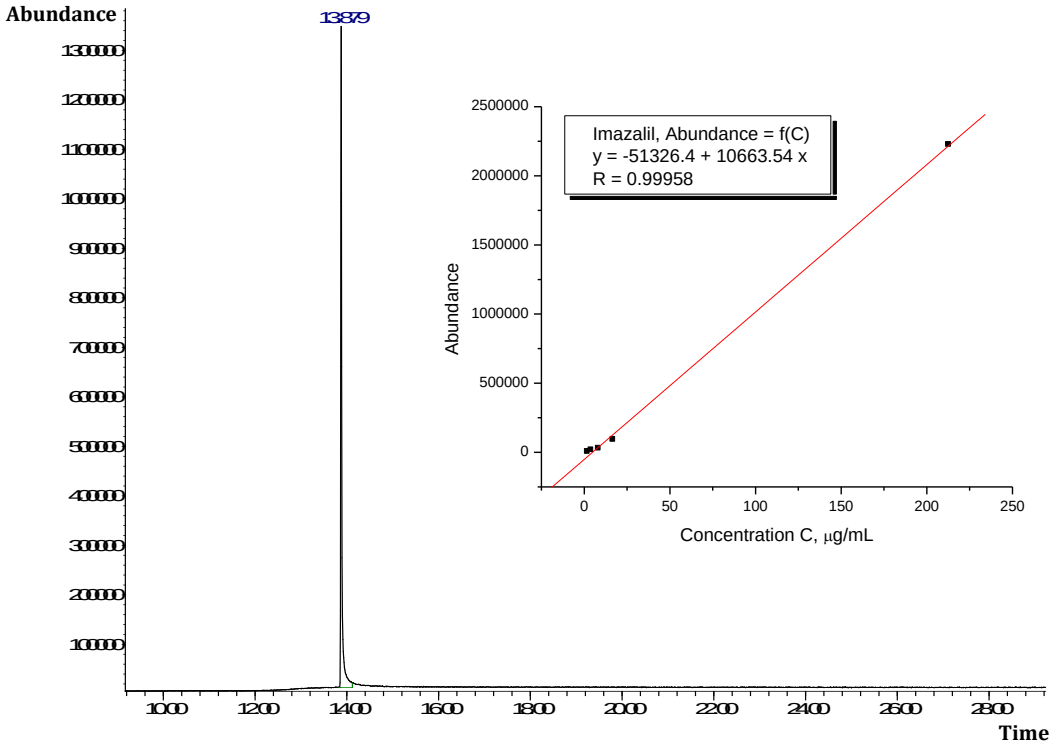

Figure 2. GC chromatogram and calibration curve of Imazalil.

Linear ranges, retention times and determination coefficients (R^2) of GC-MS and HPLC-DAD corresponding to each fungicide are shown in Tables 1 and 2.

The inferior limits for calibration curve were higher in case of GC-MS method.

Table 1. Calibration data for GC-MS analysis.

No.	Pestanal standard	Linear range, µg/mL	$t_{R.}$, min.	Observations
1.	Tebuconazole	44-1100	17.304	N=5, **R^2 = 0.9999;** SD = 1.45121E6; p<0.0001 y = -2.7099E6 + 186743.046 x
2.	Boscalid	11-57	24.358	N=5, **R^2 = 0.9912;** SD = 25855.41; p<0.0001 y = -84905.8 + 13127.6 x
3.	Iprodione	7.66-383	15.842	N=5, **R^2 = 0.9994;** SD=37713; p<0.0001 y= -79195.1 + 8219.1 x
4.	Imazalil	2.13-213	13.878	N=5; **R^2 = 0.9990**; SD=32648,6; p<0.0001, y = -51326.4 + 10663.54 x

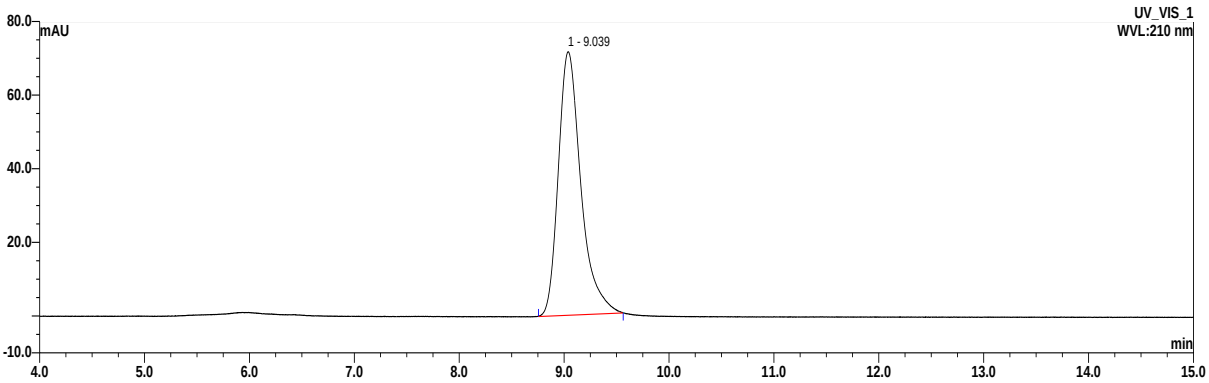

Figure 3. HPLC-DAD chromatogram of Iprodione.

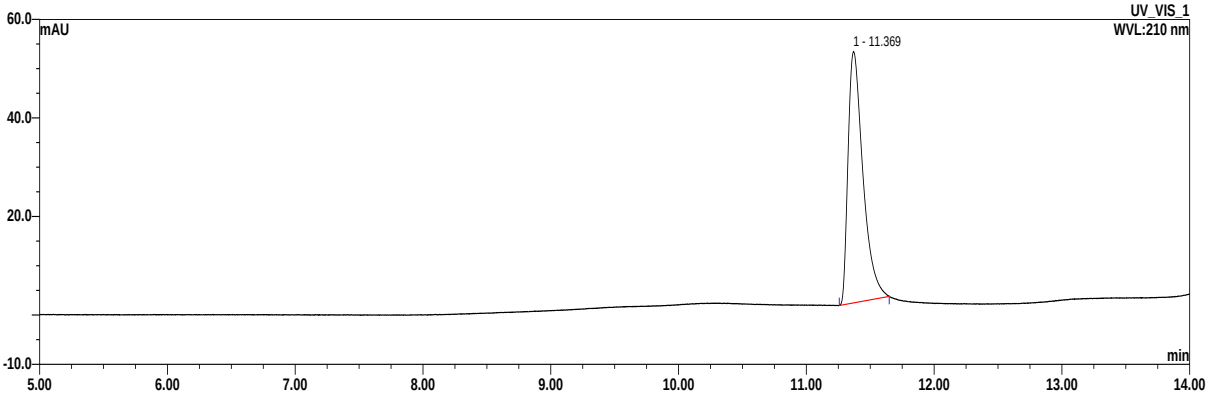

Figure 4. HPLC-DAD chromatogram of Imazalil.

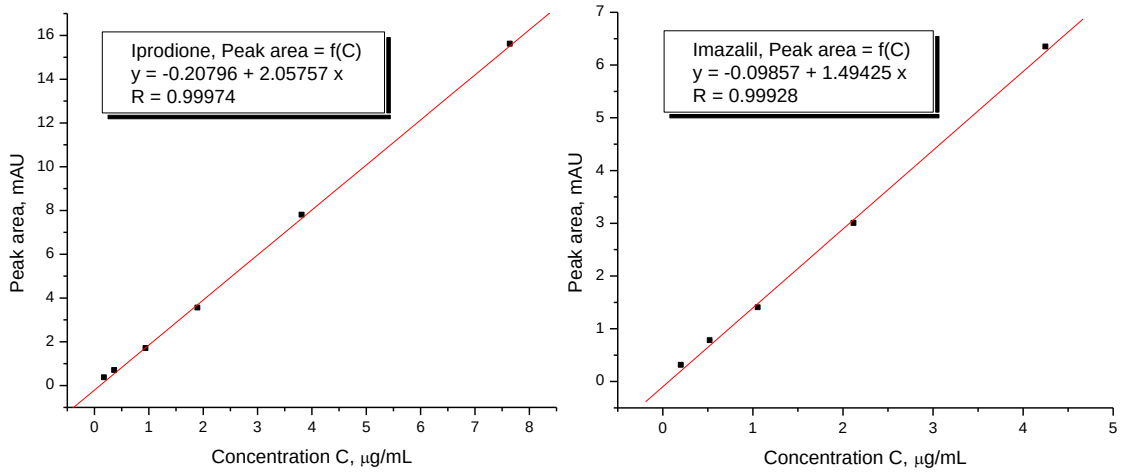

Figure 5. Calibration curve of Iprodione and Imazalil

Table 2. Calibration data for HPLC analysis.

No.	Pestanal standard	Linear range, [µg/mL]	$t_{R.}$, [min.]	Observations
1.	Tebuconazole	0.15-5.92	8.125	N=5, **R^2 = 0.9984**; SD = 0.05035, p<0.0001 y = 0.12522 + 0.45403 x
2.	Boscalid	0.28-11.28	8.533	N=6, **R^2 = 0.9999**; SD = 0.10348, p<0.0001 y = -0.02348 + 2.20999 x
3.	Iprodione	0.19-7.66	9.097	N=6, **R^2** = **0.9994**; SD=0.15042; p<0.0001 y= -0.20796 + 2.05757 x
4.	Imazalil	0.21-4.26	11.369	N=5; **R^2** = **0.9986**; SD=0.10711; p<0.0001 y = -0.09897 + 1.49425 x

The closeness of the correlation coefficient R^2 to 1 was the criteria used for assessing the linearity of the response of the studied fungicides. In all cases, R^2 was higher than 0.99, slightly higher values were obtained with HPLC technique. Tebuconazole and Imazalil had a better linear response with GC. A better linearity was obtained for Boscalid with GC, meanwhile Iprodione exhibited the same linear response with the both tested techiques.

In order to evaluate the precision of the HPLC method, the limit of detection (LOD) and the limit of quantitation (LOQ) were calculated. The LOD and LOQ values were presented in Table 3.

Table 3. LOD and LOQ values of tested fungicides (HPLC method)

Fungicide	σ	Slope	LOD	LOQ
Tebuconazole	0.00184	0.8782	0.0069	0.0210
Boscalid	0.010306	3.3743	0.0101	0.0305
Iprodione	0.01729	3.3725	0.0169	0.0513
Imazalil	0.00909	3.3996	0.0088	0.0267

Health authorities required these limits in order to appreciate and to overall understand the capabilities of any analytical method. The lower values of LOD and LOQ indicate a better fit of data.

The precision of any developed method depends on how the results may be reproduced over a period [36].

In order to evaluate the precision of Iprodione detection (Table 4), eggplants free of fungicides were contaminated with different concentration of iprodion (0.19-7.66 µg/mL).

The repeatability of the HPLC-DAD was assessed by estimating the relative standard deviation (RSD) of the detector response, expressed as peak area. In Table 4, the RSD values of Iprodione for five successive injections, at the six concentration levels, are presented. It is clear that at lower concentrations, repeatability was better, smaller RSD values were obtained in this case. It was observed that intra-day RSD $\leq$ 4.08%. The reproducibility slightly decreased between day 1 and day 3 (inter-day RSD $\leq$ 5.03%).

Table 4. Precision of Iprodione detection in eggplants.

Iprodione concentration, µg/mL	RSD %	
	Intra-day	Inter-day
7.66	4.08	5.03
3.83	3.20	3.12
1.92	3.55	3.94
0.96	0.73	3.93
0.38	2.63	2.10
0.19	1.27	2.78

Anagnostopoulos et al. (2012) analyzed more than 200 pesticides using both GC and LC techniques. Fungicides such as Iprodione (Dicarboximides) and Boscalid (Pyridines) showed better linearity and sensitivity with GC-MS-MS. For triazoles (Tebuconazole), LC-MS/MS showed better linearity, however the stability of the retention time was better in the GC-MS/MS. Imidazoles (Imazalil) showed better linearity with LC-MS/MS but better sensitivity with GC-MS/MS [37].

The levels of pesticide residues in fruits and vegetables were determined using both GC and HPLC techniques (Tabel 5).

The presence of Iprodione was detected only by HPLC method, in white and red potatoes (Fig. 6).

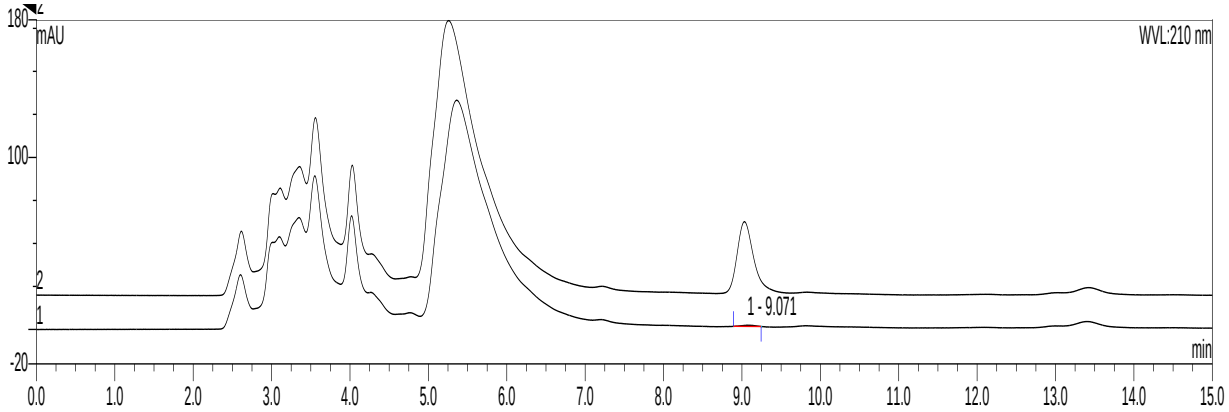

Figure 6. HPLC – DAD chromatogram of Iprodione in white potatoes: 1- white potatoes; 2- white potatoes contaminated with Iprodione.

Figs 7-9 show the chromatographic profiles of lemons and clementines. Imazalil values for lemons and clementines were slightly higher on GC-MS than HPLC. This may be due to the poor separation of Imazalil on HPLC.

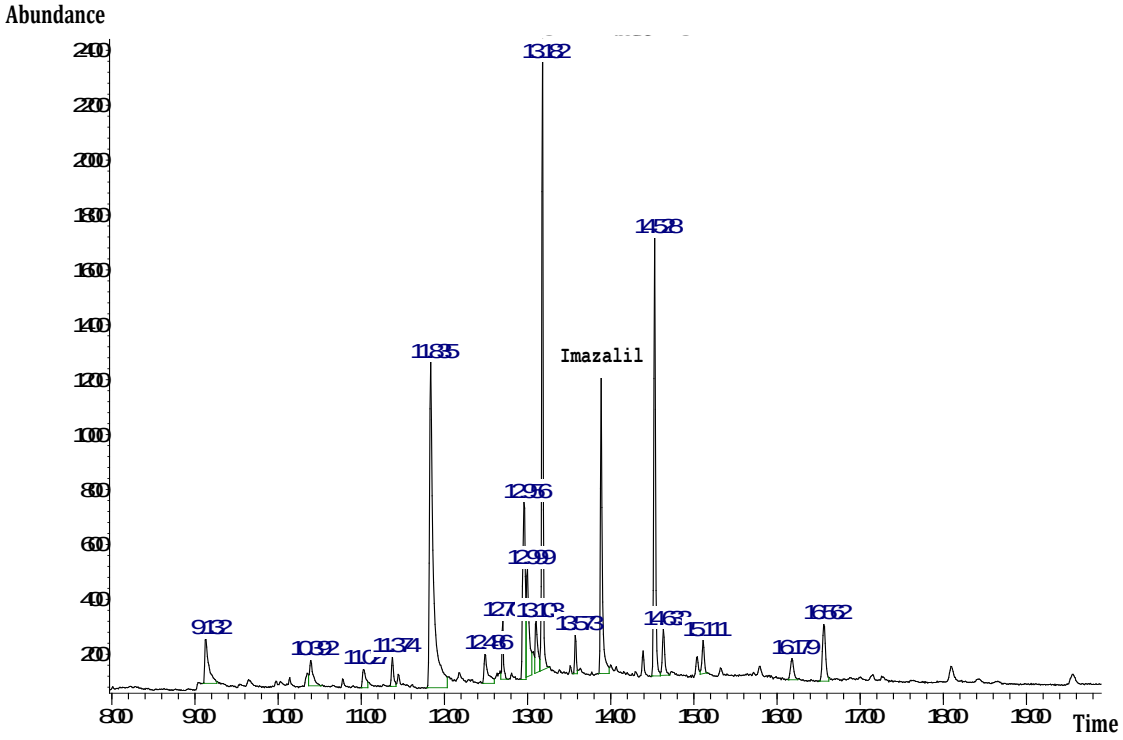

Figure 7. GC chromatogram of lemons.

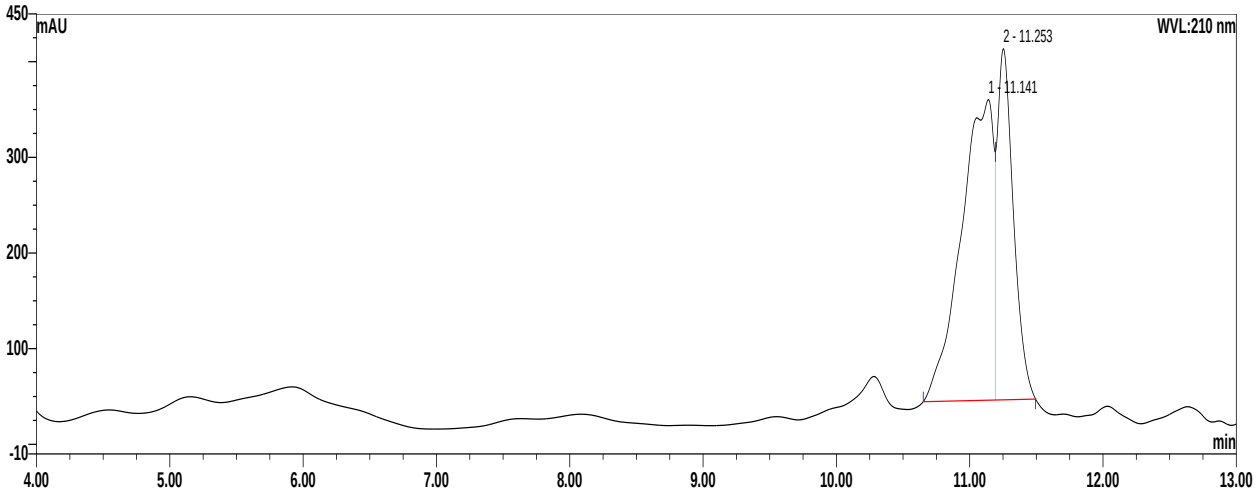

Figure 8. HPLC – DAD chromatogram of lemons.

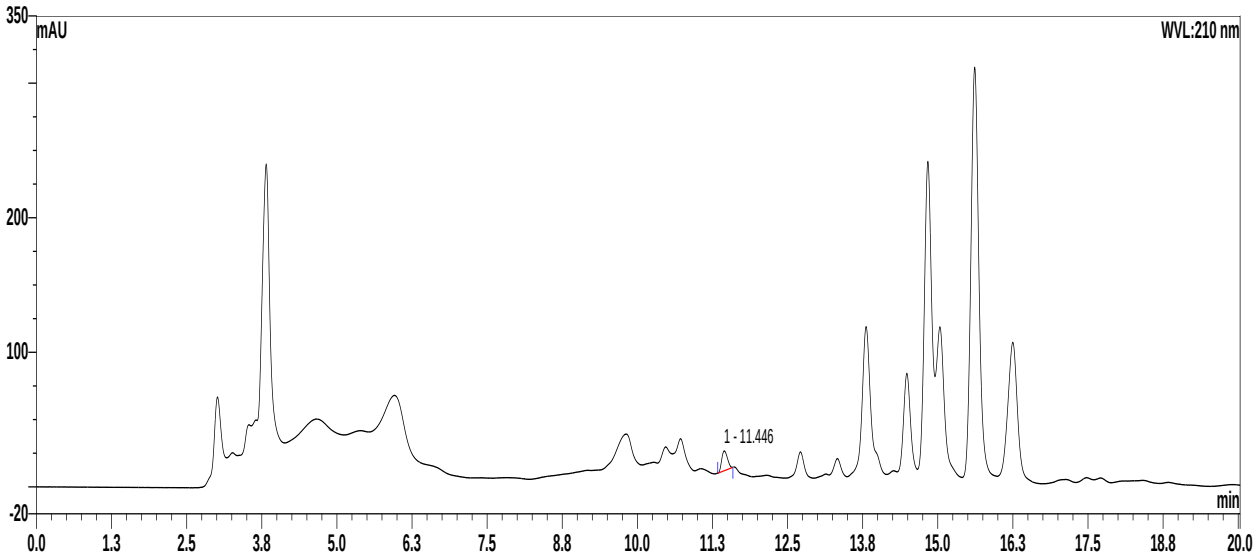

Figure 9. HPLC – DAD chromatogram of clementines.

Ortelli et al. (2005) used two multiresidue analytical methods to screen 240 samples of citrus fruits from the Swiss market. Imazalil, Iprodione and Tebuconazole residues have been found tested samples. Imazalil was detected in 70% of samples, Iprodione in 1% and Tebuconazol in 3%. Higher values (6.1 μg/g) than MRLs were obtained only for Imazalil [38]. Yamazaki and Ninomiya (1996) analyzed fifteen commercial lemon samples for Imazalil residues and found Imazalil residues between 0.2-4.5 μg/g [39].

Calvaruso et al. (2019) studied the presence of Imazalil and Boscalid, along other pesticides, in citrus fruits. Imazalil was found in the peel of the fruits in concentrations ranged between 0.008-4.5 μg/g (oranges), 3.23-4.5 μg/g (mandarines), in lemons, no Imazalil residue was found. Smaller Imazalil residue was also found in orange pulp (0.01-0.4 μg/g). As for Boscalid, this was detected only in one orange sample (0.018 μg/g), whithout being present in the fruits pulp [40].

Table 5. Fungicide amounts in studied samples.

No.	Samples	Bo [µg/g] GC	Bo [µg/g] HPLC	Te [µg/g] GC	Te [µg/g] HPLC	Ip [µg/g] GC	Ip [µg/g] HPLC	Im [µg/g] GC	Im [µg/g] HPLC
1	Eggplants, Ro	*	*	*	*	*	*	*	*
2	Cucumbers, Ro	*	*	*	*	*	*	*	*
3	Red potatoes, Ro	*	*	*	*	*	3.01	*	*
4	White potatoes, Ro	*	*	*	*	*	0.22	*	*
5	Kapia pepper, Ro	*	*	*	*	*	*	*	*
6	Plums, Ro	*	*	*	*	*	*	*	*
7	Apples, Sp	*	*	*	*	*	*	*	*
8	Lemons, Gr	*	*	*	*	*	*	26.61	25.58
9	Grapes, It	*	*	*	*	*	*	*	*
10	Clementines, Gr	*	*	*	*	*	*	1.78	1.14

**under LOQ; Bo-Boscalid; Te-Tebuconazole; Ip-Iprodione; Im-Imazalil; Ro-Romania; Sp-Spain; Gr-Greece; It-Italy*

Imazalil residues were found in clementines and lemons. Imazalil concentration in lemons was quantified, by both methods, to be higher than MRLs adopted by EU regulations [21, 22]. Iprodione was detected only with HPLC method in white and red potatoes in quantities smaller than MRLs. Boscalid and Tebuconazole were found neither in vegetables, nor in fruits.

4. CONCLUSIONS

The levels of pesticide residues in some fruits and vegetables were determined using both GC-MS and HPLC-DAD techniques. Generaly, both techniques are very sensitive and selective for the analysis of pesticides at low concentrations. GC-MS presents better linearity characteristics for Tebuconazole and Imazalil. The presence of Iprodione was detected in amounts smaller than MRLs in white and red potatoes, only by HPLC method. Imazalil residues were found in clementines and lemons by both techniques. Iprodione was detected only with HPLC method in white and red potatoes in quantities smaller than MRLs. Boscalid and Tebuconazole were not detected in studied samples.

The data obtained in this study suggest that the Romanian farmers do not longer use large quantity of fungicides, most of them being under the limits of detection. As for the imported fruits, only in lemons, Imazalil was found to exceed MRLs. Monitoring pesticide residues in fruits and vegetables offers information on the pesticide treatments that have been used till the products reach the end-consumers and helps to measure the potential risk of these products to consumer health.

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