"BABES-BOLYAI" UNIVERSITY CLUJ NAPOCA
FACULTY OF CHEMISTRY AND CHEMICAL ENGINEERING

SUMMARY

CONTRIBUTIONS TO THE DETERMINATION OF PESTICIDE RESIDUES IN VEGETABLES AND FRUITS BY VARIOUS CHROMATOGRAPHIC TECHNIQUES

SCIENTIFIC ADVISOR
Prof.dr. Constantin Măruţoiu

PhD CANDIDATE
Adela Megheşan (married Breja)

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In the summary of PhD thesis is presented in a restricted form , the own experimental results, conclusions and selective bibliography.
In summary were preserved text notation thesis chapters, subsections, figures, and tables used.

Keys : multiresidues analysis, pesticide residues, mass spectrometry, LC-MS-QQQ, GC-TOF-MS, soil, vegetables and fruits

Thesis consists in two parts.

The first part, contain the first four chapters, which presents the data in the literature regarding the current state of research in the field.

The second part of the thesis presents original experimental contributions that are presented in five chapters, the sixth being the final conclusions.

As an Annex, the results obtained with the extraction and analysis methods discussed in this paper by participating in European Proficiency Testing was presented.

INTRODUCTION

The level pesticide residues monitoring in agricultural products (vegetables and fruits), soil, water and other components of agricultural systems is a major concern for the environment.(Davidescu, Calancea, 1992)

Many studies that presents the determination of pesticide residues from other classes (harder to detect without modern devices), take in attention significant MRL's overloads for some pesticides.

However, a few studies, discusses the pesticides accumulation, their quantity in different stages of development of plants during to the periods of rest required after application of each pesticide product, pesticide residues accumulated during to the remanence time of pesticides, and identification and / or quantification of pesticide degradation products (metabolites formed) used in treatments.

This paper aims to approach all cases above, using for the study some experimental groups with different vegetables and fruits that will be treated more or less in accordance with the implementing procedures for treatment doses, sometimes "accidentally" doses from several pesticides classes: fungicides, insecticides, acaricides, will be obtained.

Pesticide residues from studied products ,will be determined using chromatographic methods: liquid chromatography mass spectrometry with triplequadrupole detector (LC-MS-QQQ), and gas chromatography mass spectrometry with time of flight detector (GC - TOF - MS).

Relevance of the project, both in terms of national monitoring programs and the European monitoring programs regarding the content of pesticides in fruits and vegetables is

given that answers of some important requirements ,regarding the pesticide exposure - health risk relationship, focusing on the determination of pesticide residues by chromatographic methods revised or newly developed, the mechanisms of action and accumulation of pesticides under repetitive application of phytosanitary treatments and the effect of overloaded pesticides doses that was applied. On the other hand, the project will produce data that want to support the idea that in modern agriculture to obtain high benefits, the pesticide selection, optimal timing of treatment and especially the application are key factors. Products with high selectivity and high economic efficiency, ecological, and nontoxic can ensure obtaining of increased crops and at the same time free of pesticides. (ICPA,2002; Davidescu, Calancea, 1992; Grou si colaboratorii , 1979)

OBJECTIVES

Considering the situation on European and national level regarding pesticide residues in various matrices, this paper proposes that the main objective ' Development of extraction and analysis methods for determination of pesticide residues in vegetables and fruits ', thereby solving other secondary endpoints, such as:

- Exposure assessment of plants in experimental field, considering the treatments that were applied in previous years of the study
- Creating an ambient experimental group and selecting crops of interest (Cherry tomatoes, Chayenne chili peppers, Cornison cucumber , Boston lettuce, grapes for wine) in order to investigate the effect of selected pesticides on plants in the manufacturer prescribed concentration taking into account the treatment technique and weather conditions
- Assessment of the effects of pesticides and their degree of accumulation in fruits and vegetables sampling in different periods of time after the plant treatments: 3 days after spraying, 10 days after spraying (after the break of the active substance), and / or 15 days or 1 month (after the break time of the active substance)
- Identification of metabolites and / or degradation products, and quantify the relevant of them

- Preparation of degradation curves representing the variation of the pesticide concentration for a certain period, and comparison with the LMA after the pesticides time break

The issues raised by this study and the objectives are closely aligned with several objectives and priorities of the SAPARD program of the European Union which had approved the establishment in 2008 of A Regional Laboratory For Determination Of Pesticide Residues In Plants And Plant Products to Phytosanitary Unit Mures from Targu Mures, which is the location where all experiments and the analysis from this paper were performed.

EXPERIMENTAL

CHAPTER 5 SELECTION OF STUDY'S AREA. PREPARATION OF EXPERIMENTAL GROUPS. CALENDAR OF APPLIED TREATMENTS. SAMPLING

The main objective of this thesis is approached in a multidisciplinary manner as a comprehensive study.

Subsections 5.1 and 5.2 presents related aspects to "Selection of the study area" and "historical soil pollution especially for experimental groups" which will make some selections. The land on which the experiment was conducted is located on the periphery of Targu Mures and has been used for growing vegetables and potatoes in last seven years. At a distance of about 100m from this land , exist another parcel uncultivated by five years, with the same soil type as the experimental field, which was used for blank vegetables samples cultivation and also for sampling of blank soil sample used to validate GC method.

This study involves four lines of investigation:

The first concerns the development of optimal extraction and analysis methods for pesticide residues suitable to achieve the intended purpose.

A second line of investigation is based on the chemical analysis of the soil chosen for the experiments in terms of pesticide content, in order to assess its quality.

A third line of investigation aims the exposure situation of selected samples to the applied pesticides in normal doses by repeated treatments with respect to a medium interval of 20 days between treatments. The data were obtained by vegetables and fruits sampling at

different time periods after the plants treatment and their chemical analysis. A series of data, those relating to historical pollution, will be collected from treatments made in previous years in selected areas and by chemical analysis of the soil in terms of the pesticide levels .

The fourth line follows assessment of metabolites resulting from the degradation of pesticides or any structural changes incurred by them during instrumental analysis using experimental investigations.

At the end of the project an integrated analysis of the data was collected.

5.3 SELECTION OF ACTIVE SUBSTANCES TO BE DISCUSSED IN THE STUDY

5.3.1 METHODOLOGY AND TECHNICAL WORK

The study was conducted during of two years, in 2012 and 2013, the pesticides of interest was grouped in three different classes, that affecting all selected samples (Cherry tomatoes, cucumbers, chili peppers , lettuce, grapes). Historical exposure to pesticides will be completed by the provided current data and by the authorities that have made previous treatments in the areas chosen for the study.

Residues of 30 pesticides in vegetables and fruits at different vegetables or fruits maturity stages, at 3, 10 and 15 days, trying to cover break times and specific remanence for used pesticide was determined. For a large number of samples were made comparative extractions. The concentration of pesticides will be reported to the maximum (MRL) possible established by the European heath state and adopted by our country as a member of it.

Selected pesticides for this study were: 2,4D, acetamiprid, thiophanate methyl, carbendazim, methomyl, imidacloprid, propiconazole, thiacloprid, cyprodinil, penconazole, dimethoate, omethoate, pyrimethanil, myclobutanil, pyraclostrobin, fenhexamid, bifenthrin, bromopropylate, captan, chlorothalonil , dicofol, folpet, iprodione, procymidone, metalaxyl, fludioxonil, fenarimol, malathion, azoxystrobin, boscalid.

The research of pesticide metabolites aims to identify any metabolites respective metabolites and quantification of some of them. Each type of sample is assumed to have a specific response after exposure to the ambient both for pesticide doses and time of metabolites appearance .

In subsections (5.3.2 and 5.3.2.1) was presented variants of plant protection products that were made treatments and most relevant physical and chemical properties of active substances under study, from the point of view of developing extraction and analysis methods.

In section 5.4 are presented "Establishing experimental groups for cherry tomatoes, cucumbers, peppers, lettuce and grapes", "sowing and pricking plants" under study " sampling technique " of vegetables, fruit and soil and "weather conditions" existing at the time of the treatment plant and sampling.

CHAPTER 6. DETERMINATION OF OPTIMAL EXTRACTION AND PURIFICATION METHODS FOR PESTICIDES FROM PLANTS AND SOIL

In this study was tested five extraction methods for vegetables and fruit, and two methods for soil. This methods was based on known methods, some of those being slightly modified in order to obtain a better extraction capacity and the accuracy of pesticides in vegetables, fruits and soil, and also to be less expensive in terms of material costs.

6.1 EXTRACTION OF PESTICIDES FROM VEGETABLES

In order to determine the optimal extraction method of pesticides from vegetables and fruits 5 known extraction methods (Luke, Klein, ethyl acetate and the original and buffered QuEChERS) were used under study, some of them being slightly modified in order to improve their efficiency depending on analyzed matrix and analysis instrument (GC-TOF-MS and LC-QQQ-MS). It was considered that the choice of a representative matrix of each group of samples classified according to Directives DG SANCO is sufficiently relevant for establishment of the optimal method of extraction group. For example, from group of matrices with "high water content" which includes tomatoes, cucumbers, peppers and lettuce etc, was chose tomatoes as a representative matrix and from group of matrices "with high acidity and high water content" as representative matrix grapes was chose. (SANCO/12495/2011, SANCO 12571/2013)

Lettuce samples were subjected to a comparative study of determination of optimal extraction method just for to highlight the efficiency of the purification method chosen for pigmented matrices.

In the following will be detailed only the methods that have been modified and were selected for carrying out the study.

6.2.1 MODIFIED MINI-LUKE

10g of each sample were accurately weighted into a 150mL polypropylene centrifuge tube and 10mL acetone was added. The mixture was shaken and then 10mL dichloromethane and 10mL petroleum ether was added. The residue was extracted using an Ultraturax homogenizer (15.000rpm for 1min) and then it was centrifuged for 5min at 4000rpm. In the case of pigmented matrices (i.e. lettuce) 7mg GBC was supplementary added. The upper organic phase (i.e. 26mL for grapes, 15 mL for tomatoes) was evaporated near to dryness on rotary evaporator at 40°C. For GC, the residue was re-dissolved in 2mL *iso*-octane:toluene 9:1, v/v, containing 0.2μg/mL HCB and filtered from 0.2 μm RC filter. For LC, the residue after concentration was re-dissolved in 2 ml acetonitrile: water 50:50, filtered through 0.2 μm filter RC and analyzed.

6.2.2 MODIFIED BUFFERED QUECHERS

10g of each sample were accurately weighted into a 100mL polypropylene centrifuge tube and were extracted with 10mL acetonitrile acidified with 1% acetic acid. After the addition of 200μl from a 10 μg/ml HCB solution, the residues were extracted using an Ultraturax homogenizer (15.000 rpm for 1min). Then, 4g $MgSO_4$, 1g NaCl and 1g CH_3COONa were added and the resulting mixture was shaken for 1min and then centrifuged for 5min at 4000rpm. An aliquot of the acetonitrile extract (6mL) was transferred to a 20mL polypropylene centrifuge tube and 150mg PSA and 900mg $MgSO_4$ were added. For pigmented matrices 15mg GBC was additionally added. The mixture was shacken by hand for few seconds, filtered from 0.2 μm RC filter and then 1 ml of extract was concentrated under N_2 steam. The residue was re-dissolved in 1mL *iso*-octane:toluene 9:1, v/v ,filtered from 0.2 μm RC filter and analyzed by GC.

For LC analysis, after stirring for 1 minute and centrifuging at 4000rpm for 1 minute, 1 ml of a mixture acetonitrile: water 50:50 was added on 1ml of purified acetonitrile extract, then all mixture was filtered from 0.2 μm RC and analyzed .

6.2.6 SELECTION OF OPTIMAL METHOD FOR PESTICIDES EXTRACTION IN FRUITS AND VEGETABLES

To compare the performance of extraction methods under study, blank samples of each representative matrix (tomatoes, grapes and lettuce) were spiked with a mixed solution at 50µg / kg containing 80 pesticides. From all identified metabolites was decided to quantify five of them: tetrahydrophthalimide, thiophosgene - metabolites of captan, phthalimide - metabolite of folpet, 2.4 '-diclorobenzofenonă - metabolite of fenarimol and dicolfolului and 4,4 diclorobenzofenonă- metabolite of dicofol.

Comparison of extraction methods is illustrated graphically in Figure 6.2 on two intervals recovery rates (under 70% and over 120%), the results of Klein extraction method (DIN EN 15637) are not represented because only 30 pesticides were quantified, and only 15 pesticides have been identified.

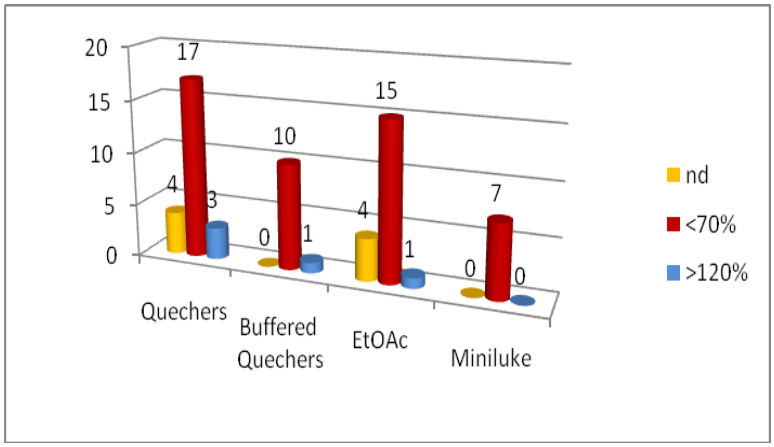


Figura 6.2 Comparison of pesticides number within two recovery range (<70% and >120%) or not detected (n.d) at 50 µg/kg spike level on grapes samples.

Comparative recovery rates for extracted pesticide metabolites by 4 extraction methods was presented in table 6.1.

Table 6.1 Comparative recovery rates for extracted metabolite pesticides by 4 extraction methods

Metaboliţi	QuEChERS	QuEChERS tamponat	EtOAc	Mini-Luke modificat
2,4'-Diclorobenzofenonă	0	69	0	59
Ftalimidă	0	41	0	73
p,p'-Diclorobenzofenonă	123	113	0	42
Tiofosgen	0	30	70	107
Tetrahidroftalimida	0	47	0	57

After analyzing all of obtained data was concluded that the modified Mini-Luke extraction method and buffered QuEChERS extraction method provided acceptable results, for which these two extraction methods will continue to be used to finalize the objectives proposed in this paper. For extraction of pesticide residues and their metabolites from 5 matrices (cherry tomatoes, cucumbers, chilies, lettuce and grapes) will be establish which from these two methods is optimal (from each matrix) associated, of course ,with the analysis method. (Megheşan – Breja, Măruţoiu, Cimpoiu, acceptat pentru publicare 2014)

2,4 DICHLOROPHENOXYACETIC ACID (2,4D) EXTRACTION METHOD

The alkaline hydrolysis with NaOH or K2CO3 can achieve an increase of its extractability from fruits and vegetables due to breaking of links that may exist between this pesticide and matrix. The proposed method for 2,4-D extraction from tomatoes is: 10 g of the accurately weighed sample is placed in a 250 ml centrifuge tube made of polypropylene. Add 2 ml of 10M NaOH was stirred vigorously for 1 min and then allowed to stand for 30 min. Then it was neutralized with 5 mL of 3M H2SO4 and 15 mL ACN with 1% CH3COOH was added and then stirred in the Ultraturax (1min at 15.000rpm). After that 3 g CH3COONa dry, 3 g NaCl , 6 g MgSO4 was introduced, for 1 min was hand shaked, and then was centrifuge for 5 minutes at 4000 rev / min. From the resulting extract (about 12 ml) 1 ml was take it, and 1 ml ACN: H2O in the ratio 50:50 was added; filtered through 0.2 μm RC filter and analyzed by LC.

6.4 EXTRACTION OF PESTICIDES FROM SOIL

One of those two methods described below has been selected as optimal extraction method of pesticides from soil,and that was method with PSA, which has the advantage that the number of the extracted pesticides are greater ,and have better recovery rates and the fact that the extract is more "clean", which enabled the analysis of a greater number of samples of soil without the need to inject acetone to clean the liner or GC injector.

6.4.1. EXTRACTION METHOD WITH PSA

10 g of soil sample were accurately weighted into a 250 mL polypropylene centrifuge tube and mixed with 10 mL water and 20 mL acetonitrile acidified with 1% acetic acid. After the addition of HCB (0.2µg/mL), the residues were extracted using an Ultraturax homogenizer (15.000rpm for 1min). Then, 4g $MgSO_4$, 1g NaCl and 1g CH_3COONa were added and the resulting mixture was shaken for 1min and then centrifuged for 5min at 4000rpm. An aliquot of the acetonitrile extract (6mL) was transferred to a 20mL polypropylene centrifuge tube and 150mg PSA and 900mg $MgSO_4$ were added. The mixture was shacking by hand for few minutes, centrifuged and then 1 mL from the resulting extract (ca 4 mL) was analyzed by GC TOF-MS.

6.5 VEGETABLES AND FRUITS EXTRACTS PURIFICATION

In the present study, in the case of the samples that containing more pigments, such as lettuce, for Mini- Luke extraction method a GBC purification step was introduced.

In order to evaluate the efficacy of the purification, the blanks (grapes, tomatoes and lettuce) was spiked at a concentration level of 0.05 to 0.1 mg / ml. The lower values of the rates of recovery for some compounds with planar structure is a consequence of the use of GBC purification step (it retains this compounds from the samples) and also probably due to the concentration extract step.

According to DG DANCO 12571/2013 recommendations , in this paper was decided to use HCB as internal standard injection, so it is finally adding, with the dissolving extract solution , this decision being supported by the fact that if it is added before sample extraction, in purification step with GCB, HCB due to its planar structure will be retained by it.

Some briefly conclusions of the GBC purification step are as follows:

- In the extraction procedure with EtOAc , purification step with GBC should be avoided as pesticide loss was very high; only 10% of active substances were found after purification and recoveries rates for quantified pesticide were between 30-55% .

- Extraction Mini-Luke was applied to lettuce, cherry tomatoes and grapes. After purification, the recoveries were between 55-161%. During the analysis of unpurified extract of lettuce have been encountered the following problems: clogging of the injection syringe, the height and abundance of m / z of pesticides peaks decreased or increased, retention time modification, the last ones being produced by the matrix effects which involved in the analysis.

- In the extraction with ACN, the influence of the matrix components (i.e. essential oils and pigments) was lower than for the other extraction methods. The loss of pesticide from GBC purification step were significant, but comparable to those obtained using modified Mini-Luke with GBC purification step.

- The obtained recovery rates indicate that the modified Mini-Luke extraction method is more suitable for the extraction of pesticide residues from various samples excluding pigmented and oil samples, for such tests is preferred QuEChERS (ACN) extraction method.

- The matrix effect was evaluated by comparing the peaks area obtained from the standard solutions of pesticides (n = 3) , with those obtained from the lettuce extract(blank) spiked to the same pesticides.

- Similar results were obtained in the case of soil samples, which extracts was purified by SPE technique, using PSA and mixed salts .

- In the case of LC analysis , the GBC purification step was applied only for the lettuce sample and just modified Mini-Luke and buffered QuEChERS extraction methods were investigated. Only 4 pesticides (carbendazim, imidacloprid, thiophanate methyl and methomyl) from 17 pesticides investigated had higher recovery rates for by Mini-Luke extraction method than by buffered QuEChERS extraction method.

CHAPTER 7. DEVELOPMENT OF METHODS FOR DETERMINATION OF PESTICIDES FROM PLANTS AND SOIL

7.1 DEVELOPMENT OF GC -TOF- MS ANALYSIS METHOD

7.1.1 MATERIALS AND METHODS

The analysis of this study are performed with a modified AGILENT 6890 series gas chromatograph (have two ovens), coupled with a LECO Pegasus (USA) mass spectrometer, an autosampler and split / splitless injector AGILENT 7683 series. Development of GC-TOF-MS analysis method has started in 2008 after a team training of LZPDRPPPV chemists (of which I was part) with a team of analysts from the European Technical Centre in Prague, belonging of the producing firm LECO (USA). Initially, 60 pesticides were analyzed with that method, and in the course of years, the method is almost completely changed and the number of pesticides was reached to over 100 . The GC method presented in this thesis is modified to the original method (GC settings) , only certain parameters of the processing method and MS method, were kept, these parameters are mostly recommended by the manufacturer as being optimal. So the developed method from this paper started with the separation of 104 pesticides and will make a selection of 30 pesticides from these for applied study (vegetables and fruits treatments). As a result, it will be presented the selected pesticides for applied study , but will make numerous references to the other pesticides that can be determined with this method. Analytical standards and reagents are all HPLC and GC certified and are purchased from Sigma –Aldrich company. The used salts have GC purity and are purchased from Merck company; cartridges, adsorbent materials PSA and C18, GBC and 0,2μm RC filters are purchased from Supelco company. Ultrapure water was prepared with a device consisting of two units TKA Lab tower and GenPure2.

Other used equipments: MettlerTolledo and Kern balances, Grindomix and Ultraturax homogenizers, Heidolph rotary evaporator, N-EVAP116 OA-SYS nitrogen evaporator current(USA), Consul20 centrifugal, Sonorex ultrasonic bath, Hirshmann Laborgerate dispensers.

7.1.2 THE STUDY OF PHYSICO-CHEMICAL CHARACTERISTICS OF THE ACTIVE SUBSTANCES

The studied pesticide active substances are grouped in several chemical classes, but have the

different physico-chemical properties even if they are of the same chemical groups, such as water solubility, polarity, vapor pressure, melting point .

7.1.3 FACTORS THAT INFLUENCING SEPARATION

In this section was presented a number of factors that can affect the chromatographic separation of pesticides in gas chromatographic analysis, with examples from the situations that arising during to the development of analytical methods GC-TOF-MS. Factors such as point boiling the polarity of the stationary phase from the column versus the polarity of the analytes, the temperature of the injector ,oven, temperature ramps, the length and internal diameter of the column, the flow rate of the carrier gas, the quantity of sample injected, ion source temperature, the rate of acquisition of spectra, can affect the separation , some of them is shown in more detail in the following.

7.1.3.4 OVEN TEMPERATURE COLUMN. TEMPERATURE RAMP

The column temperature is a factor in gas chromatography that can change area and height of a chromatographical peak in various proportions. A high temperature shortens the retention time, but this is in the disadvantage of separation since most compounds are in the gas phase and to be separating, the compounds must to interact with the stationary phase, otherwise there will be no marked differences in times retention. The best separation is obtained with a program of the oven temperatures, because the polarity and boiling point will intervene into the separation. (Fernandez-Alba, 2012)

In view of the literature observations, in a first attempt was used an initial oven temperature of 100 ° C, and the temperature ramps are shown in Figure 7.1.

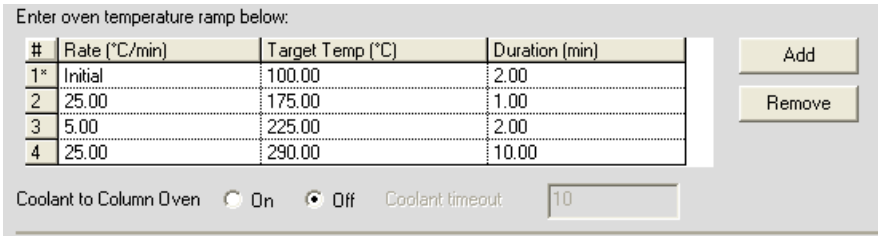


Figure 7.1 Selection of the temperature ramp, Test 1

However, in order to separate the isomers of certain pesticides as well as the high boiling compounds and some contaminants, was built a more complex temperature ramp. This following ramp was a total analysis time of 29.2 minutes and separate 92 pesticides.

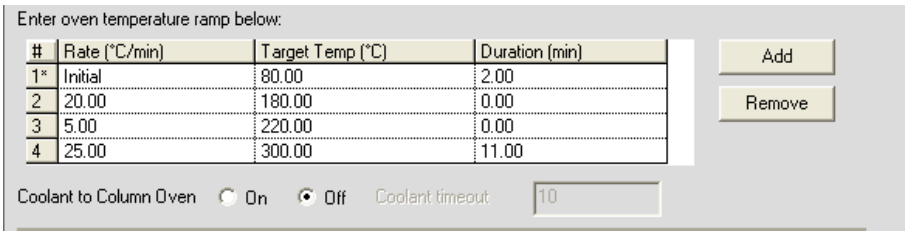


Figure 7.2 Selection of the temperature ramp, Test 2

In figure 7.3 are some pesticides, from a mixture injected on a 0.5 ppm concentration with all other parameters unchanged, except GC temperature ramps, in order to support what has been said above.

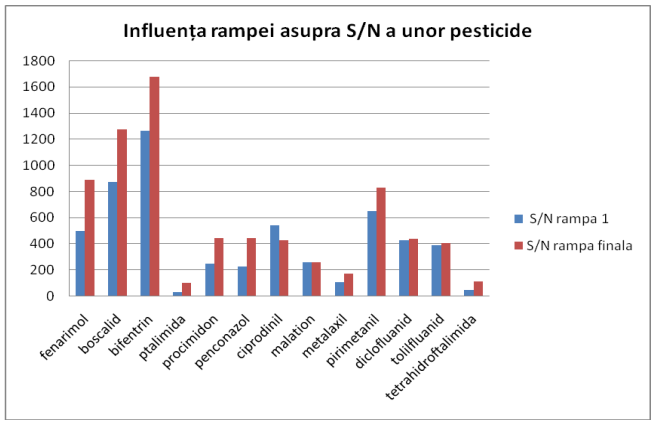


Figure 7.3 Temperature ramps influence on the S / N of pesticides

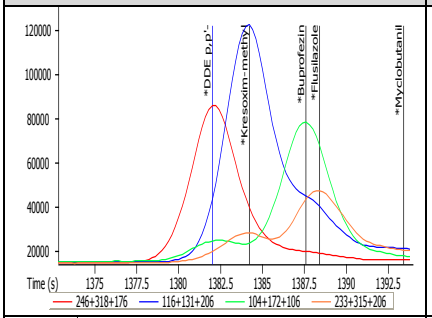
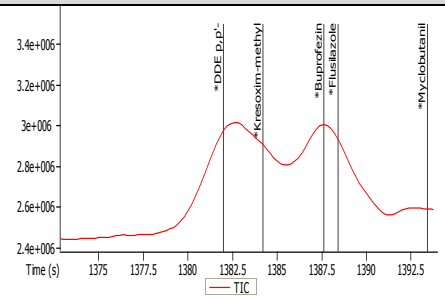
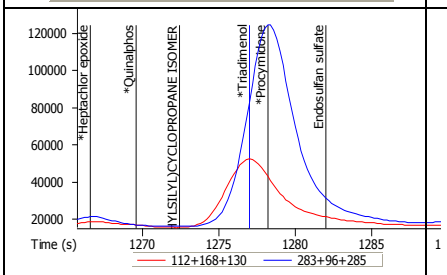
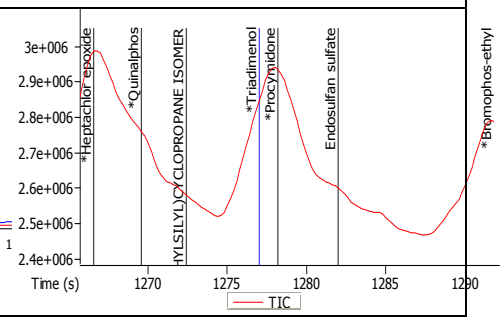
7.1.3.6 CARRIER FLOW RATE

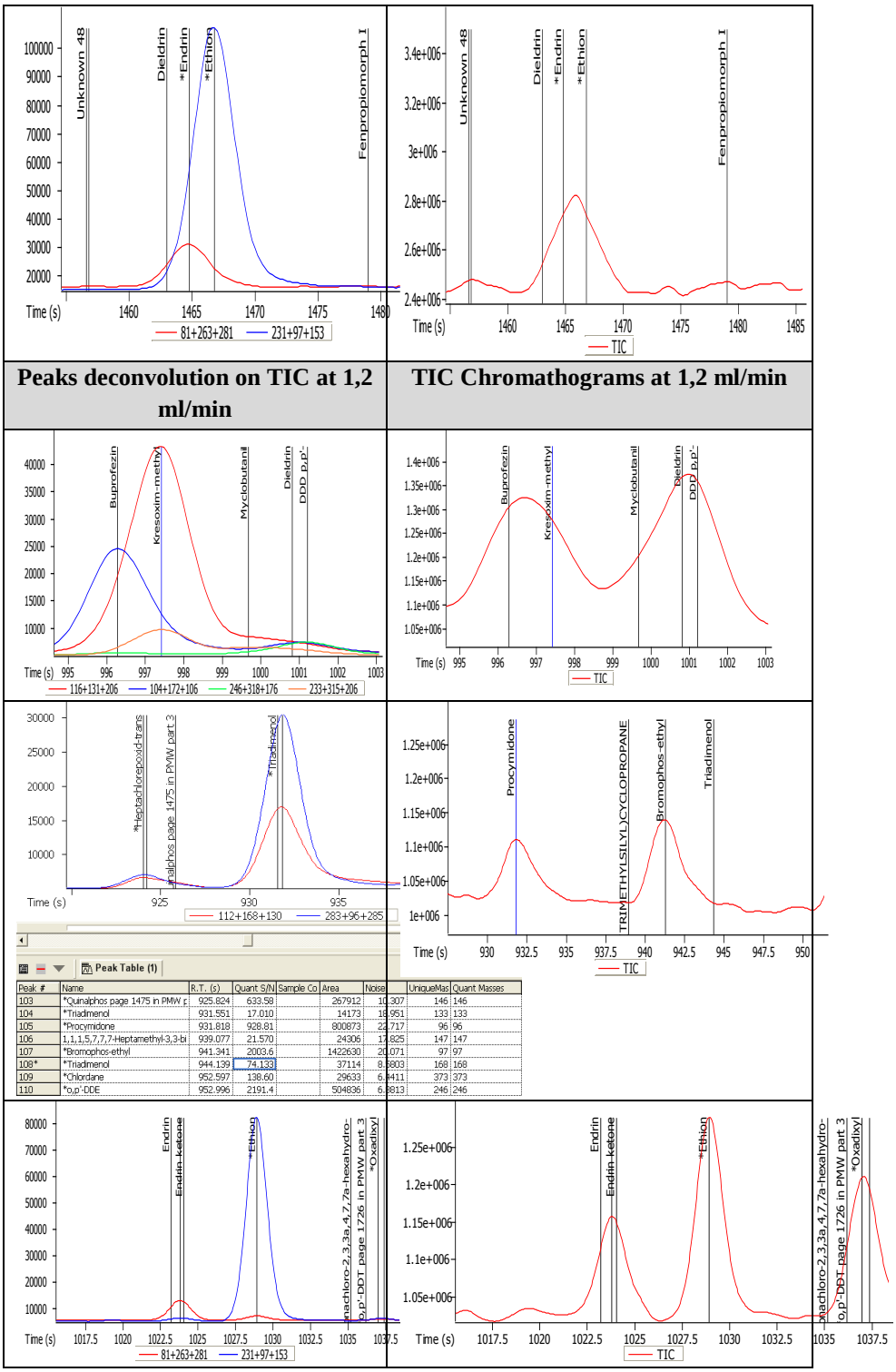
A high flow rate of carrier gas (helium in this case), reduces the retention time of the analyte and produces a poor separation and a low flow rate of the carrier gas increases the retention time. (de Koning and Gumpendobler, 2007)

After the last setting of temperature ramps was passed to the establishing of the optimal carrier flow rate. It began with a flow rate of 1.0 ml / min, and then changed to 1.2 ml / min, all

other GC parameters being kept unchanged. The most important reason for this change the flow was that were observed co-elution of pesticides and have tried a better separation of peaks. In Figures 7.4, 7.5 are shown three examples of pesticide behaves differently to the changing of the carrier flow rate. Should be noted that even the peaks are not well separated in terms of chromatography, they are separate and integrated through the deconvolution algorithm of GC-TOF-MS instrument from LECO.

As it seen some peaks are well separated by increasing of carrier flow rate (eg. Ethion, endrin) other was part separated (eg. Kresoxim-methyl, buprofezin remained coeluted ,but flusilazole and pp 'DDE were separated) while others remained unseparated, but appeared isomers of some of them (eg. triadimenol remains coeluted with procymidone, but appears isomer 2 of triadimenol of which can be quantified; this isomer was not even identified at flow rate of 1.0 ml / min).

Peaks deconvolution on TIC at 1,0 ml/min	**TIC Chromathograms at 1,0 ml/min**
	
	



7.1.4 FINAL CONDITIONS FOR GC-TOF-MS ANALYSIS

An LECO Pegasus Time-of-Flight Mass Spectrometer (TOF-MS) (USA) equipped with an Agilent 6890 series gas chromatograph with two ovens, Agilent 7683 series Autosampler and

a split/splitless capillary injector port, was used. Chromatographic separation was achieved on two capillary columns, first RXi-MS 30m x 0.25mm x 0.25µm (Restek, USA) and second BPX50 1.6m x 0.1m x 0.1µm (SGE Analytical Science, Australia). The injector temperature was 250°C and splitless injection was performed using helium as carrier gas with a flow rate of 1.2mL/min. The ovens temperatures were programmed as follow: oven 1 - 80°C (2 min), 20°C/min to 180°C (0 min), 5°C/min to 220° C (0min), 25°C/min to 300°C (11min); oven 2 - 110°C (2 min), 20°C/min to 210°C (0 min), 5°C/min to 250°C (0 min), 25°C/min to 330°C (10min) , without modulation. The injection volume was 1µL. The mass spectrometer was operated in electron ionization mode (EI) and full scan mode monitoring between m/z 40 and m/z 450, with ionization energy of 70eV and acquisition rate of 15 spectra/second. The transfer line temperature was kept at 280°C.

In the processing method and MS method is specified a delay time for acquisition at 350s, to remove the solvent; rate matching has been set at least 70% and was used 10 pesticides spectra libraries; the detector voltage has been set at 1700V, which in time can be increased to 2000V depending on the operating time of the detector.

Figure 7.6 illustrates the temperature ramps for two ovens ,transfer line and total analysis time (29.2 minutes).

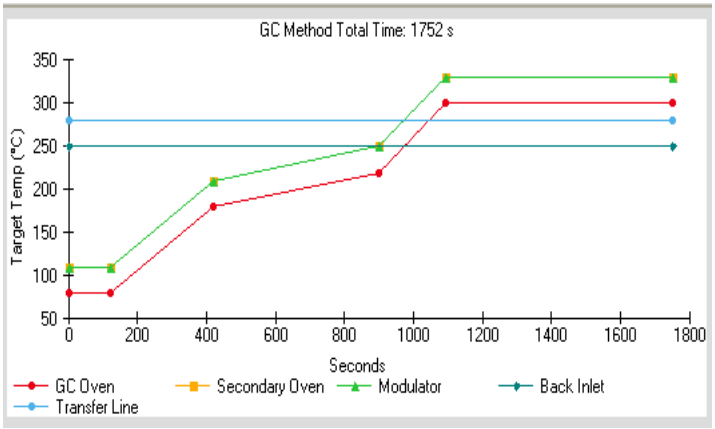


Figure 7.6 Temperature ramps of the two ovens, and the transfer line .Total analysis time (29.2 minutes)

7.2 DEVELOPMENT OF METHOD FOR DETERMINATION OF PESTICIDES IN VEGETABLE PRODUCTS BY LC-MS-QQQ

7.2.1 THE STUDY OF PHYSICO-CHEMICAL CHARACTERISTICS OF ACTIVE SUBSTANCES

Pesticides that were analyzed by LC : 2,4D, azoxystrobin, pyraclostrobin, boscalid, thiophanate methyl, omethoate, dimethoate, carbendazim, acetamiprid, fenarimol, penconazole, cyprodinil, pyrimethanil, malathion, imidacloprid, methomyl thiacloprid, propiconazole, hexythiazox, cymoxanil, many of them as secondary substances in plant protection products used for treatments.

Unfortunately , cymoxanil can not be analyzed , because the analytical standard used for identification and quantification was completely degraded, in addition in many papers who studied this pesticide , cymoxanil was classified as undetectable as a result of its structure.

7.2.2 MATERIALS AND METHODS

LC analysis were performed with an AGILENT liquid chromatograph equipped with a quaternary pump model 1200, autosampler and equipped with a mass spectrometer triple quadrupole 6410 AGILENT, ionization source type Multi mode ionization (MMI), ionization type ESI and APCI. In developing LC-MS-QQQ method was worked only on ESI ionization and was started with the consultation on a wide variety of methods, focusing on some methods from the manufacturer (Agilent) who used various mobile phases and modifiers. Finally, taking into account the optimal parameters in order to achieve a good separation chromatography, it was considered appropriate to be selected for working method , the reverse stationary phase for chromatographic separation (Zorbax XDB C18 column) and as mobile phase a mixture of ACN and water ultrapure 0.1% formic acid as modifier. (Thurman , Ferrer, 2005; 2008)

Certain parameters for MMI ionization source and ESI ionization are specified by the manufacturer to establish a certain value, such as nebulizer pressure should always be 60 psi, drying gas flow always maintained at 5 l / min. (Agilent Guide User, 2008)

In the next subsections were presented several factors that governing retention in reversed phase chromatography, some of them will be presented in more detail in this summary of the

thesis, with examples of situations encountered in the development of LC-QQQ-MS analytical methods.

7.2.3 INFLUENCE OF FLOW PHASE SEPARATION ON MOBILE

To study the influence of mobile phase flow rate were kept all other parameters, only varying flow. Taking into account the column manufacturer's instructions was tested flow rates of 0.6, 0.5 and 0.4 ml / min, finally opting for 0.4 ml / min flow rate. Even if peaks seems to be the same for all flow rates studied, with 0.4 ml / min flow rate ,most of the obtained peaks have large areas and heights , this factors being important in the LOD and LOQ determination. For a better example ,Table 7.2 presents comparative pesticide peak areas analyzed at rates of 0.6 and 0.4 ml / min.

Table 7.2 The influence of mobile phase flow rate on separation

Pesticide	**Peak Area flow rate 0.4 ml/min**	**Peak Area flow rate 0.6 ml/min**
Methomil	92414	91313
Carbendazim	168433	172844
Pyrimetanil	77292	78110
Omethoate	47686	43418
Acetamiprid	252490	253549
Thiophanate methyl	551260	593632
Boscalid	5642	5277
Fluquinconazole	911	917
Pyraclostrobin	738841	650800
Azoxystrobin	1043487	895050
Dimethoate	178809	175322
Thiachloprid	216047	209082
Imidacloprid	186883	183300
Malathion	18814	19538
Fenarimol	99090	104899

7.2.4 INFLUENCE OF MOBILE PHASE COMPOSITION ON SEPARATION

Considering the indications in the literature, have made a number of changes to obtain a gradient elution scheme for an acceptable separation for all analyzed pesticides.

Here is one of the results of the optimization gradient (Figure 7.8). Omethoateand carbendazim tend to co-elute (have near log P values) in first separation test, then modifying the gradient steps was obtained a fine separation of the two pesticides, but there are other peaks of other pesticides that were from the start in the solution, but were identified just before changing the gradient steps. Pesticides that have near log P values , and especially polar, which were eluted first, was a real challenge in terms of obtaining a good chromatographic separations.

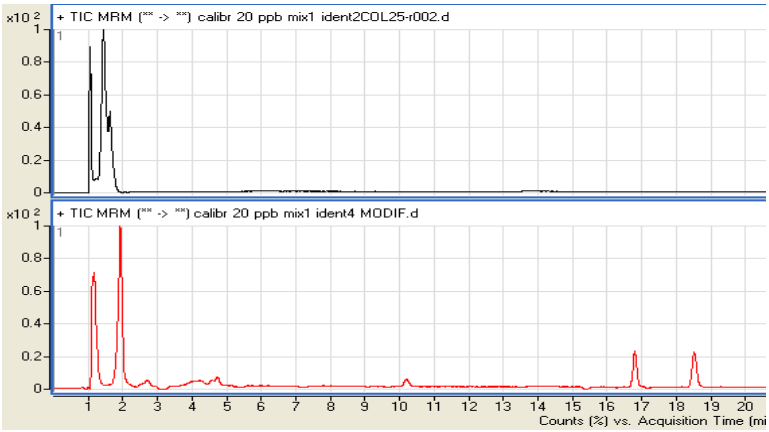


Figure 7.8. The influence of the gradient in the separation of a pesticides mixture

The final elution gradient is as follows:

	Time	B%	Flow	Max. Press.
1	0.00	20.0	0.400	400
2	28.00	80.0	0.400	400
3	30.00	100.0	0.400	400
4	31.00	100.0	0.400	400
5	33.00	20.0	0.400	400
6	35.00	20.0	0.400	400

7.2.5 INFLUENCE OF pH MOBILE PHASE ON SEPARATION

pH control in the RP-LC is carried out by acid or buffer solutions. It's use buffer solutions based on sodium acetate- acetic acid or ammonium formate - formic acid.

Figure 7.10 was presented three changes of the mobile phase composition which was made on LC method development, with a focus on the separation of the first eluted pesticides peaks ,

which have close polarities and were difficult to separate. In the first chromatogram of separation is shown having mobile phase A: water with modifier 0.1% formic acid + 5 mM ammonium formate and B: acetonitrile; The second has as mobile phase A: water with modifier 0.1% formic acid, and B: acetonitrile with modifier 0.1% formic acid; the third chromatogram has as mobile phase A: water with modifier 0.1% formic acid, and B: acetonitrile. It should be noted that for each change was presented the best separation chromatograms of first eluted pesticides, and the injected pesticides mixture was the same concentration in all cases.

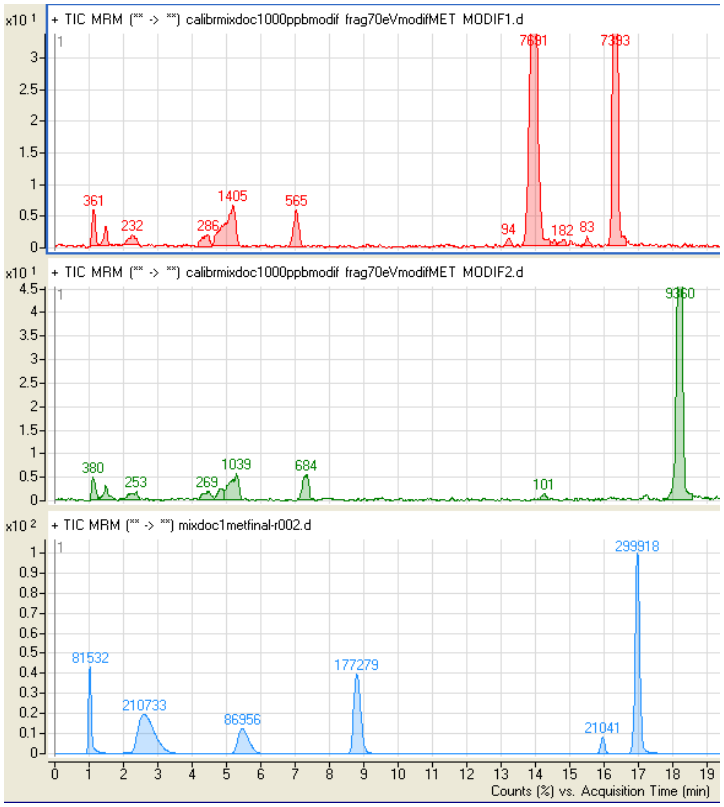


Figure 7.10 Comparative study of pH change of mobile phase by introducing modifiers (formic acid and ammonium formate)

7.2.6 INFLUENCE OF CERTAIN PHYSICO-CHEMICAL PROPERTIES OF ACTIVE SUBSTANCES ON SEPARATION

The active substances can be grouped into several groups according to the values of the partition coefficient octanol / water (log P). The gradient elution program and grouping of pesticides in separate sets of analysis so as to not be in the same set more than two pesticides

which may have co-eluted to achieve a good chromatographic separation. In figure 7.11 TIC chromatograms are shown and these was obtained with the same analysis method performed under the same conditions: A-mixture contain 25 pesticides at a concentration of 100 ppb in a single injection; B, C, D, E mixtures contains 3-8 pesticides each, the same as in mixture A at a concentration of 100 ppb, but the separately injected, so that finally if it's collect, also 25 pesticides peaks were obtained.

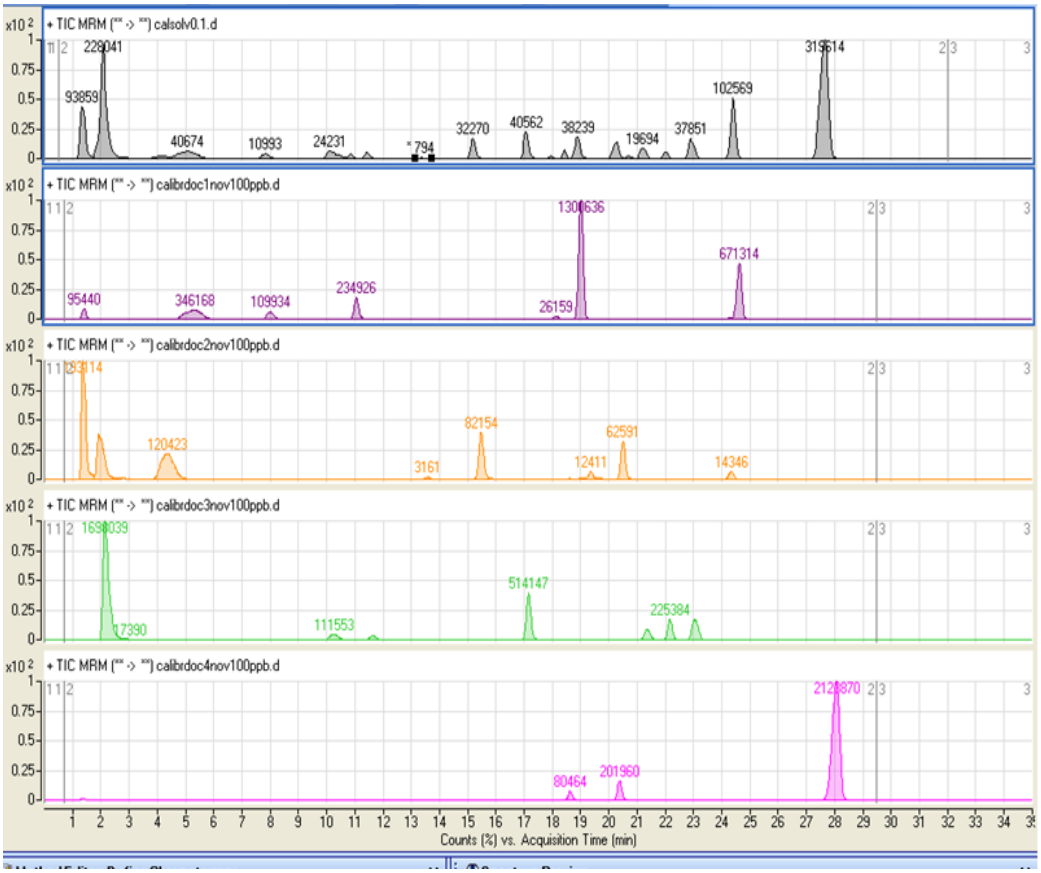


Figure 7.11 The influence of the number of injected pesticides on one separation

Immediately noticeable are the peak areas and peak amplitude obtained in separate groups from these separations. Also peaks shape is superior to those obtained at the injection mixture of 25 pesticides. Can be easily seen the co-elution in the separation A at RT 1-2, approx. 4, but placed in separate groups can bring an improvement in quantitative analyzes. It should be noted also that large peaks obtained both in the chromatogram A and in other represents 2 co-eluted

pesticides and in the case of chromatogram A , 3 pesticide co-eluted at RT about 4 (acetamiprid, imidacloprid, methomyl).

7.2.7 FINAL CONDITIONS FOR DETERMINATION OF PESTICIDE RESIDUES BY LC-MS-QQQ

Final developed method to determine pesticide residues in vegetables and fruits by LC-MS-QQQ has the following parameters:

LC parameters:
Chromatographic column: Zorbax Eclipse XDB-C18, 1.8 micron 4.6x50mm
Flow rate of mobile phase: 0.4 ml / min
Column temperature: ambient (25 ° C)
Injection volume: 10μL
Mobile phase: A: water + 0.1% formic acid; B: Acetonitrile
Elution gradient:

Time	min B%
0	20
28	80
30	100
31	100
33	20
35	20

MS parameters:
Ionization source : MMI
Ionization mode: ESI positive
Energy Fragmentation: 70-120 V- specific of each pesticide
Energy Collision5-20 eV - specific pesticide of each pesticide
Capillary voltage: 2500V
The temperature of the gas in the ion source: 350 ° C
Nebulizer pressure 60 psi
Drying gas flow 5 L / min
Gas for nebulization, desolvation and collision: nitrogen

7.3 DEVELOPING THE MONORESIDUAL ANALYSIS METHOD FOR 2.4 D BY LC-MS-QQQ

2,4-D is a selective herbicides, used to protect cereals against broadleaf weeds. It is also applied as growth regulator during plant growth (tomatoes), and post-harvest for fruits protection , especially citrus fruits.

Tomatoes is considered one of the most sensitive crops in terms of 2,4-D and its derivatives. Like the growth stimulatory was applied in sub-lethal doses ranging from 0.42 - 13.44 g / ha directly to the plants in different growth stages from the beginning of flowering.

7.3.1 MATERIALS AND METHODS

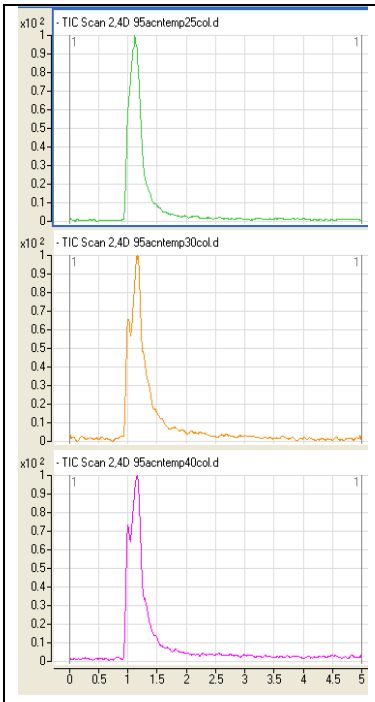
Analysis instrument is a liquid chromatograph Agilent with a quaternary pump Agilent serie 1200 , autosampler, and coupled with a mass spectrometer Agilent 6410 QQQ triple quadrupole with MMI-type ion source (Multi Mode Ionization).
In the following subsections was treated several factors during development of LC method with examples, this factors can influence the determination of 2,4D pesticide.

7.3.4 INFLUENCE OF TEMPERATURE ON SEPARATION

Temperature is an important parameter of chromatographic separation. It's very good illustrated the influence of temperature in the 2,4D analysis in figure 7.14 , where can be compare the peaks shapes according the different values of temperatures (25, 30 and 40 ° C); the final selected temperature for 2.4D analysis was set at 25 ° C. [203]

7.3.5 INFLUENCE ON SEPARATION OF INJECTED SAMPLE VOLUME

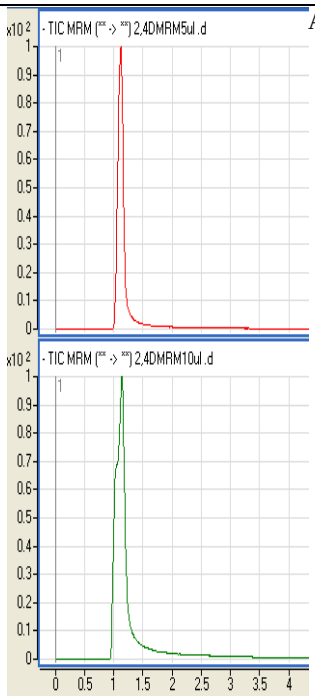
The obtaining of some splitted or broad peaks has a determinant factor the injected sample volume.



A.Oven temperature 25°C

B.Oven temperature 30°C

C. oven temperature 40°C



A. injection volume 5µL

B. injection volume 10µL

7.3.7FINAL CONDITIONS FOR 2,4D RESIDUES DETERMINATION BY LC-MS-QQQ

Column: Zorbax Eclipse XDB-C18, 1.8 micron 4.6x50mm
Mobile Phase:
B: 95% ACN + 0.1% HCOOH
A: 5% H2O + 0.1% HCOOH
FM rate: 0.5 ml / min, temperature isocrat
Column temperature: ambient (25 ° C)
Ionization type: negative ESI

Capillary voltage: 2500V
The gas temperature in the ion source: 350 ° C
Nebulizer pressure 60 psi
Drying gas flow 5 L / min
Gas for nebulization, desolvation and collision: nitrogen
Fragmentation energy: 70 eV
Collision energies:
transition 219 → 161, 10eV
transition 219 → 125, 25eV
Injection volume: 5µL
Total analysis time: 3 minutes

CHAPTER 8. VALIDATION OF MULTI-RESIDUES GC AND LC ANALYTICAL METHODS FOR DETERMINATION OF PESTICIDE RESIDUES IN VEGETABLES, FRUITS AND SOIL

In this chapter was presented the validation of the analysis and extraction developed methods in various matrices as follows:

• GC-TOF-M method with Mini-Luke extraction method was validated for tomatoes and grapes

• GC-TOF-MS method with PSA extraction method was validated for soil

• LC-QQQ-MS method with modified QuEChERS extraction method was validated for chili peppers

8.1 VALIDATION CRITERIA FOR UNDER DG SANCO GC APPLIED TO VEGETABLES, FRUITS AND SOL

Although soil is not placed as matrix in DG SANCO guide, the validation of GC method for soil were made to take into account the requirements of DG SANCO / 12495/2011. In accordance with the guidance of DG SANCO 12495/2011 and DG SANCO 12571/2013 implemented at 01/01/2014 were checked following validation criteria for each representative matrix of matrices groups according to DG SANCO, including for soil and for each analyte : linearity, matrix effect, LOD, LOQ, specificity, repeatability, reproducibility, measurement uncertainty.

Conclusions for performed validations are presented briefly below.

After GC analysis method with modified Mini-Luke extraction method validation on tomatoes and grapes , can punctuate a few conclusions:

- 67 pesticides from 85 pesticides proposed for method validation on tomatoes was validated, only those fulfilling all the parameters of acceptability according to DG SANCO / 12495/2011

  • 61 pesticides from 85 pesticides proposed for method validation on grapes was validated, only those fulfilling all the parameters of acceptability according to DG SANCO / 12495/2011

  - 60 from 88 pesticides proposed for GC method with specific PSA extraction validation on soil, was fully validated .

- LC analysis method with modified QuEChERS extraction method has been validated on chillies for 28 from 32 pesticides proposed for validation.

CHAPTER 9. DETERMINATION OF PESTICIDE RESIDUES IN VEGETABLES AND FRUITS

Spraying technique was "taken" from the technique application mode of most people without a qualification in plant protection that treats its plants more often than recommended dose and excessive spraying them (actually bathe them) using a hand mini-sprayer. In this study, the used doses were consistent with those recommended by the manufacturer of plant protection products. However, the fact that the excess of phitosanitary product will be applied to the plant, even at the recommended dosage, will have the effect of achieving higher concentrations of active substances (pesticide).

Samples were quantified by calibration curves in solvent, for each pesticide being presented calibration curve equation or calibration curve. The pesticides concentration from sample extractions that exceeds the maximum concentration from the calibration curve were diluted with re- dissolving solvent, and the dilution factor was specified for each pesticide.

The results are presented as comparative charts covering analysis technique (GC and / or LC) and extraction method (modified Mini-Luke and / or modified QuEChERS) for each pesticide. Also, graphics representing the pesticide degradation values obtained with different extraction and analysis at 3, 10 and 15 days was built.

9.1 DETERMINATION OF PESTICIDE RESIDUES IN TOMATOES CHERRY

9.1.1 GC -TOF-MS MULTI-RESIDUES METHOD

In the 2012 were studied in Cherry tomatoes matrix, the following pesticides: fenarimol, azoxystrobin, boscalid, malathion, cyprodinil, penconazole, dimethoate, pyrimethanil, bifenthrin, bromopropylate, captan, folpet, chlorothalonil, dicofol, iprodione, procymidone, metalaxyl. In 2013 was added to the study two other pesticides: fludioxonil, myclobutanil. Samples were quantified by calibration curves in solvent isooctane: toluene 9: 1, for each pesticide being presented calibration curve equation or calibration curve. The pesticides concentration from sample extractions that exceeds the maximum concentration from the

calibration curve were diluted with re- dissolving solvent, and the dilution factor was specified for each pesticide. The results was reporting in mg / kg and was applied a correction factor depending on the method and the organic extract volume remaining after all processing steps, but in the comparative results graphs the concentrations was expressed in ppb.

The calibration curves were constructed in seven calibration levels 0,01-0,03-0,06-0,09-0,27-0,54-0,81 mg / ml.

The analysis sequence was established from injections at each calibration level , followed by the analyzed tomatoes samples, a blank sample spiked at 0.05 µg/ml (50 ng/ml) for each extraction methods and a new series of injections from calibration levels to the ends. Thus, each calibration level was constituted by 2 injection points.

Each pesticide is identified and quantified by 3 m / z ratio , with a similarity with libraries spectra at least 70% and also for each one was determined the optimum extraction method using the recovery rates obtained, GraphPad InStat statistical program by applying a statistical calculation (t test pair), just for guidance and in some cases was consulted LOQ values and chromatographic peak shapes obtained.

9.1.2 LC-MS-QQQ MULTI-RESIDUES METHOD

In 2012 the study were subjected Cherry tomatoes matrix, the following pesticides: fenarimol, azoxystrobin, boscalid, malathion, cyprodinil, penconazole, dimethoate, pyrimethanil, omethoate, acetamiprid, thiophanate methyl, imidacloprid, methomyl, carbendazim.
In 2013 they added the study of other pesticides: fenhexamid, myclobutanil, propiconazole, pyraclostrobin, thiacloprid.

Samples were quantified by calibration curves in solvent acetonitrile: water 50:50, for each pesticide being presented the calibration equation in Table 9.1.2. The pesticides concentration from sample extractions that exceeds the maximum concentration from the calibration curve were diluted with re- dissolving solvent, and the dilution factor was specified for each pesticide. The results was reporting in mg / kg and was applied a correction factor depending on the method and the organic extract volume remaining after all processing steps, but in the comparative results graphs the concentrations was expressed in ppb.

For pesticides carbendazim, boscalid, cyprodinil, malathion, imidacloprid, methomyl calibration curves were built on five calibration levels at 0,01-0,025-0,05-0,1 and 0.25 µg / ml. For

pesticides thiophanate methyl, pyraclostrobin, azoxystrobin, fenarimol, propiconazole, thiacloprid, acetamiprid, dimethoate, omethoate, calibration curves were built on five calibration levels at 0,01-0,025-0,05-0,14 and 0, 25 μg / ml. For pesticides myclobutanil, pyrimethanil and fenhexamid calibration curves were built on four calibration levels at 0,02-0,05-0,1 and 0.4 μg / ml, and for penconazole the calibration curve was built 5 calibration levels, respectively 0,006-0,015-0,03-0,06 and 0.15 mg / ml.

The analysis sequence was formed from an injection at each calibration level followed by analyzed tomatoes samples, a blank sample spiked at 0.05 μg / mL for each extraction method, and finally another injection for each calibration level . Thus, each level is constituted by 2 injection points. Each pesticide is identified and quantified by two transitions (one for quantification and the second for confirmation), and also for each pesticide was determined which extraction method is optimal using recovery rates obtained for each pesticide, and applying just as guidance a statistical program GraphPad InStat with statistical calculation (t test pair).

9.1.3 COMPARATIVE STUDY 2 methods of extraction / 2 methods of analysis

Two extraction methods (modified QuEChERS and modified Mini-Luke) and two analytical methods (GC-TOF-MS and LC-QQQ-MS) were compared. Defining for the selection of the most effective extraction and analysis method was recovery rates obtained from spiked blank samples at 0,05μg / ml and processed by those two extraction methods, if was necessary peak shapes and amplitudes was compared and just for guidance was applied a statistical program GraphPad InStat, ANOVA- Bonferroni test (analysis of variance). Pesticides from Cherry tomatoes analyzed with 4 combination (extraction method / analysis method) were fenarimol, azoxystrobin, boscalid, malathion, cyprodinil, penconazole, dimethoate, pyrimethanil, myclobutanil, fenhexamid and pyraclostrobin.

9.1.4.8 CONCLUSIONS - PESTICIDE RESIDUES IN CHERRY TOMATOES

The conclusions on the effectiveness of combinations used in this matrix are formulated briefly as a table.

Table 9.1.3 The efficiency of extraction and analysis methods for pesticides in Cherry Tomatoes, according to the study conducted

LC QuEChERS modif	LC Mini-Luke modif	GC QuEChERS modif	GC Mini-Luke modif
acetamiprid thiophanate methyl carbendazim methomyl imidacloprid propiconazole thiacloprid cyprodinil penconazole dimethoate pyrimethanil myclobutanil pyraclostrobin fenhexamid	Tiofanat metil Carbendazim Fenarimol Azoxistrobin Boscalid Penconazol	Bifentrin Bromopropilat Dicofol Folpet Iprodion Procimidon Fludioxonil Ciprodinil Pirimetanil Miclobutanil Fenhexamid	Bifentrin Bromopropilat Captan Clorotalonil Dicofol Folpet Iprodion Procimidon Metalaxil Fludioxonil Fenarimol Malation Pirimetanil

In this matrix were identified and quantified five metabolites of pesticides (tetrahydrophthalimide, phthalimide, 4,4'diclorobenzofenonă, thiophosgene, omethoate) and other 10 metabolites have been just identified, but was presented only the most important and that had a great similarity with spectra libraries: 3,5 dichloroaniline, p,p' dibromobenzophenone, 2,4 dichlorobenzophenone, 3,4 dichlophenyl isocyanate.

Regarding to the MRL, taking into account the treatment conditions of the plant and climatic conditions, a number of five pesticides were exceeded the MRL recommended values.

9.1.5 SINGLE RESIDUE METHODS LC-MS-QQQ FOR 2.4 D IN CHERRY TOMATOES

In this study was desired to observe the persistence of the 2,4 D herbicide as growth stimulator and, for this purpose was prepared a solution with a concentration of 0.2% to the product amine salt known as DMA in which was soaked tomato flowers and green tomato fruits, the last treatment being approximated to be made before 2 weeks of ripening. Tomato samples were sampling after full ripening of fruits, were milled and stored at -18 ° C. Unfortunately could not be made the analysis of these samples (malfunction of equipment auxiliary LC -nitrogen generator), but was developed LC analytical method, built calibration curves in solvent and

matrix and was analyzed three blank samples fortified at 0.5 μg / ml for calculation of recovery rates.

Calibration was performed on 5 levels solvent calibration, at 0,016-0,048-0,096-0,288-0,576 μg / ml, two injections for each calibration level. Calibration in matrix was performed on 5 calibration levels, lower than the solvent concentration at 0,0099- 0,0247- 0,0495-0,148-0,297 μg / ml, each calibration level consisting from 3 injections.

Recovery rates that was obtained (65%) are satisfactory in the way of extraction of the pesticide and other results reported in the literature, of which are from undetected (Pizzutti, 2009) to 104% (Klein & Alder, 2003) in various matrices including tomatoes.

9.2 DETERMINATION OF PESTICIDES IN CUCUMBER

9.2.1 GC -TOF- MS MULTI-RESIDUES METHOD

In 2012 were studied in the cucumber matrix, the following pesticides: fenarimol, azoxystrobin, boscalid, malathion, cyprodinil, penconazole, dimethoate, pyrimethanil, bifenthrin, bromopropylate, captan, folpet, chlorothalonil, dicofol, iprodione, metalaxyl.

In 2013 was added to the study other 3 pesticides: fludioxonil, fenhexamid and pyraclostrobin.

Calibration curves were built from seven calibration levels at 0,01-0,02-0,05-0,1-0,2-0,5-0,8 mg / ml.

Analysis sequence was composed by one injection to each calibration level, followed by analyzed cucumber samples and in the end with a blank samples spiked at 0.05 μg / ml (50 ng / ml) for each of the two extraction methods, so that each calibration level is constituted by a single injection point.

Quantification and identification of pesticide in samples was performed in the same way as in the cause of Cherry tomatoes.

9.2.2 LC-MS QQQ MULTI-RESIDUES METHOD

In 2012 were studied in the cucumber matrix the following pesticides: fenarimol, azoxystrobin, boscalid, cyprodinil, penconazole, dimethoate, omethoate, acetamiprid, thiophanate methyl, imidacloprid, methomyl, carbendazim.

In 2013 was added to the study 3 other pesticides: fluquinconazole, pyraclostrobin, thiacloprid

Calibration curves were built on 5 levels, the levels for penconazole 0,006-0,015-0,03-0,06 and 0.15 μg / ml, and for all other pesticide levels were 0,01-0,025-0,05-0,1-0,25 μg / ml. Sequence of analysis was formed from an injection at each calibration level followed by analyzed cucumbers samples and finally a blank sample spiked at 0.05 μg / mL for each extraction method.

Quantification and identification of pesticide in samples was performed in the same way as in the cause of Cherry tomatoes.

9.2.3 COMPARATIVE STUDY (2 EXTRACTION METHODS / 2 ANALYSIS METHODS)

In the comparative study were analyzed the following pesticides: fenarimol, azoxystrobin, boscalid, cyprodinil, penconazole, dimethoate and pyraclostrobin. Calibration levels and sequence of analysis are similar to those described above for the pesticide determined by LC and GC and the quantification of the samples and identify the pesticide was made in the same manner as for Cherry tomatoes.

9.2.5 CONCLUSIONS - PESTICIDE RESIDUES IN CUCUMBER

Have been identified and quantified four metabolites of the pesticide which was used for plant protection treatments, respectively, tetrahydrophthalimide, phthalimide, 4,4dichlorobenzophenone and omethoate.

MRL's have been exceeded for six pesticides : bromopropylate, captan, folpet, dicofol, procymidone and thiophanate methyl.

The conclusions regarding the effectiveness of analysis /extraction combinations used in this matrix are formulated briefly as a table.

Table 9.2.8 Efficient extraction and analysis methods for pesticides in cucumber, according to the conducted study

LC QuEChERS modif	LC Mini-Luke modif	GC QuEChERS modif	GC Mini-Luke modif
Acetamiprid	Fluquinconazol	Pirimetanil	Malation
Tiofanat metil	Dimetoat	Dicofol	Bifentrin
Imidacloprid		Metalaxil	Bromopropilat
Carbendazim		Fludioxonil	Captan

Fluquinconazol Fenarimol Azoxistrobin Boscalid Ciprodinil Penconazol Piraclostrobin		Procimidon Fenarimol Boscalid penconazol	Folpet Clorotalonil Iprodion Ciprodinil

9.3 DETERMINATION OF PESTICIDES IN CHILLI PEPPERS

9.3.1 GC -TOF-MS MULTI-RESIDUES METHOD

The study was conducted during of the two years 2012 and 2013, and as test matrix chilies was used the Chayenne variety , being analyzed the following pesticides: fenarimol, azoxystrobin, boscalid, cyprodinil, penconazole, dimethoate, bromopropylate, captan, folpet, chlorothalonil, fludioxonil.

Calibration curves were constructed in four calibration levels at 0,01-0,05-0,2-0,8 μg / ml. Sequence of analysis was formed from an injection at each calibration level followed by the analyzed chili peppers samples, a blank sample spiked at 0.05 μg / ml (50 ng / ml) for each of the two extraction methods and finally another injection at all calibration levels, so each calibration level is made up of 2 injection points.

Quantification and identification of pesticide samples was performed in the same manner as the matrice samples above.

9.3.2 LC-MS-QQQ MULTI-RESIDUES METHOD

In 2012 and 2013, during which this study was conducted, in chili peppers Chayenne matrix were analyzed the following pesticides: fenarimol, azoxystrobin, boscalid, cyprodinil, penconazole, dimethoate, methomyl, fluquinconazole, pyraclostrobin, thiophanate methyl, acetamiprid, fenhexamide, omethoate , imidacloprid, carbendazim, myclobutanil.

Calibration curves were built on five calibration levels at 0,01- 0,04 - 0,1- 0,2 - 0,4 μg / ml. The analysis sequence was formed from an injection at each calibration level, followed by the analyzed samples and finally a blank sample spiked at 0.04 μg / mL for each extraction method.

9.3.3 COMPARATIVE STUDY (2 EXTRACTION METHODS / 2 ANALYSIS METHODS)

Pesticides that was analyzed comparative in chili peppers matrix were cyprodinil, boscalid,

penconazole, fenarimol, dimethoate, azoxystrobin. The calibration levels of the analysis sequence are similar to those described above for the pesticide determined by LC and GC and the quantification of the samples and identify of pesticides was made in the same manner as in the above matrix.

9.3.5 CONCLUSIONS - PESTICIDE RESIDUES IN CHILLI PEPPERS

Four metabolites of pesticides used to treat chili peppers have been identified and quantified and other three metabolites were identified but without sufficient data to quantify them: 1,2,4 triazole (myclobutanil or fluquinconazole metabolite) identified in RT 1190 ,3s and RT 649,5s, glycine at RT 427,98s possible breakdown product of cymoxanil, (pesticide which was not analyzed, but which were used for chili peppers treatment) and the acid metabolite of azoxystrobin.
In terms of compliance with MRL, 4 pesticides had values above the established European standards in the field, but should be considered both the conditions of treatment and climate. Some of these conclusions can be found in a restricted form in Table 9.3.9.

Table 9.3.9 Efficient extraction and analysis methods for pesticides in chili peppers Chayenne, according to the conducted study

LC QuEChERS modif	LC Mini-Luke modif	GC QuEChERS modif	GC Mini-Luke modif
Methomyl Pyraclostrobin Acetamiprid Imidacloprid Carbendazim Myclobutanil Cyprodinil Boscalid Azoxistrobin	Cyprodinil Penconazole	Folpet Brompropylate Fludioxonil Dimethoat	Chlorothalonil Captan Boscalid Fenarimol

9.4 DETERMINATION OF PESTICIDES IN LETTUCE

This matrix was introduced in the study of extraction and purification with GBC step methods as a result of the high content of chlorophyll in the organic extracts obtained after extraction, whatever it may be the extraction method. Therefore for this matrix was performed only one phitosanitary treatment, after which were taken samples at 3 and 10 days.

GC instrumental analysis was performed on both purified and unpurified extracts, the difference between the results of some quantitied pesticide was very high, due to the retention of them to the GBC. The LC analysis was performed for one sample without purification (the samples that was sampled at 3 days , in order to avoid clogging the chromatographic column and contaminate the MS system , all the others samples being purified by GBC.

For quantification of pesticide residues in lettuce were used the same calibration curve in solvent as for the chili peppers, these two matrices together with the grapes were examined in the same sequence, both for GC and LC.

Calibration levels and identification of pesticide was made in the same way as for the matrix chili peppers.

9.4.1 GC- TOF- MS MULTI-RESIDUES METHOD

Pesticides analyzed by GC are as following: bifenthrin, captan, folpet, bromopropyl, boscalid, penconazole and dimethoate.

9.4.2 LC- MS-QQQ MULTI-RESIDUES METHOD

By LC analysis in lettuce were analyzed the following pesticides: acetamiprid, methomyl, pyraclostrobin ,thiophanate methyl, boscalid, penconazole, and dimethoate

9.4.3 COMPARATIVE STUDY (2 EXTRACTION METHODS / 2 ANALYSIS METHODS)

The comparative study analyzed pesticides: boscalid, penconazole, and dimethoate

9.4.5CONCLUSIONS - PESTICIDE RESIDUES IN LETTUCE

MRL values were exceeded in 4 cases, for some of the pesticides was suggested a break time because they were not approved for this matrix, but it can be found accidentally as a result of the

plant treatments in greenhouses, that can comprising many other cultures that for the plant protection products are approved.

In the lettuce samples was not aimed primarily to quantify the identified metabolites because these came from active substances that was not approved for lettuce; these metabolites was: tetrahydrophthalimide and phthalimide. Table 9.4.5 presents some of the above conclusions.

Table 9.4.5 Efficient extraction and analysis methods for pesticides in lettuce, according to the study conducted

LC QuEChERS modif	LC Mini-Luke modif	GC QuEChERS modif
Pyraclostrobin Penconazole	Acetamiprid Thiophanate methyl Boscalid Dimethoat	Biphentrin Captan Bromopropylate Folpet

9.5 DETERMINATION OF PESTICIDES IN GRAPES

Analyses were carried out in 2013, using as the only QuEChERS method extraction method for LC analysis and both extractions for GC analysis. In august, one treatment with specific products was applied in time when the grapes were 30% ripe. GC method validated for grapes was used for the determination of pesticides in a variety of white wines from our country; the Mini-Luke modified extraction method was used to extract pesticide residues in wines, these results are not presented in this thesis, but are the subject of an article. To quantify the residues of pesticides in grapes were used the same solvent calibration curve as in the chili peppers, these two matrices together with the lettuce were examined in the same sequence, both for GC and LC. Calibration levels and identification of pesticide are the same as the chili peppers matrix.

9.5.1 LC-MS- QQQ MULTI-RESIDUES METHOD

Pesticides under study by LC analysis method was fenhexamide, thiophanate methyl and hexythiazox, the last one can not be quantified due to too high LOQ value.

9.5.2 GC-TOF-MS MULTI-RESIDUES METHOD

Pesticides cyprodinil and procymidone were determined by GC-TOF-MS method, these two pesticides was the most successful to combat gray mold (Botrytis cinerea) on grapes.

CHAPTER 10. GENERAL CONCLUSIONS

This thesis has set as its main objective the development of extraction and analysis methods to determine the relationship between the accumulation, degradation and recovery of pesticides in the samples under study. The problem is approached in a multidisciplinary manner as a comprehensive study.

The issues raised by this study and the objectives are closely aligned with several objectives and priorities of the European Union SAPARD program which aimed the establishment in 2008 of a Regional Laboratory for Determination of Pesticide Residues in Plants and Plant products to Phytosanitary Unit Mures, from Targu Mures, which is the location where all experiments and the analysis were conducted for this thesis.

The most important innovations the thesis are:

- Development of new extraction and analysis methods and / or modify existing ones in order to simplify and increase the performance parameters (analysis methods)
- Elaborate the analysis methods validation on certain matrices and their use in European proficiency testing (shown in appendix)
- Exposure matrix approach for chosen fruits and vegetables at 30 pesticides from several chemical classes, to the normal concentration by repeated treatments
- Obtaining exposure data by analyzing residues of active substances (pesticides) by chromatographic techniques, GC-TOF-MS and LC-QQQ-MS
- Obtaining data regarding metabolites and degradation products resulting from metabolic processes or during the analysis of pesticides

- In order to determine the optimal extraction method were tested five extractive methods for fruits and vegetables, and two methods for soil. The methods was based on known extraction methods: QuEChERS buffered ,QuEChERS original, Mini Luke, ethyl acetate, DIN EN 15637 (Klein), some of them being slightly modified
- After analyzing of all the obtained data it was concluded that the Mini-Luke modified extraction method and buffered QuEChERS modified extraction method provided acceptable results, for which these two extraction methods were used to remove 30 pesticide residues and their metabolites from 5 matrices (Cherry tomatoes, cucumbers, chili peppers, lettuce and grapes)
- GC analysis method was validated according of DG SANCO / 12495/2011 on a large number of pesticides to those selected for the study of pesticide residues in fruit ,vegetable and soil samples: 60 pesticides in soil, 67 pesticides in tomatoes, 61 pesticides in grapes, among them being the pesticides used for treatments on field experimental crops
- LC analysis method was validated for 28 pesticides in chili peppers, more than in the samples analyzed by LC, among them being the pesticides used for treatments
- The concentration of pesticide residues determined after passage of break time was reported at MRL values established by the European Union and adopted by our country as a member of it.
- Metabolites and / or degradation products were identified and the most relevant were quantified (tetrahydrophthalimide, phthalimide, 4,4'diclorobenzofenona, thiophosgene, omethoate)

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