STUDIA UBB CHEMIA, LXVII, 4, 2022 (p. 249-259)
(RECOMMENDED CITATION)
DOI:10.24193/subbchem.2022.4.16

DETERMINATION OF 2,4-DICHLOROPHENOXYACETIC ACID (2,4-D) FROM TOMATOES BY LC-MS/MS ANALYSIS

Liana Maria COSTEA[a], Raluca RAD[b], Claudia CIMPOIU[b], Adela MEGHESAN[b,*]

ABSTRACT. 2,4-D is a herbicide that in certain doses can be used as a growth stimulator for various crops. Although it is forbidden in our country to use it for such purposes, tomato growers use it to obtain large and fast harvests, but unfortunately of poor quality (the well-known tomatoes with tassel). In this work, we present a modified Miniluke extraction method and a LC-MS/MS analysis method of this pesticide from tomatoes. The method was validated both on tomatoes (presented in this paper) and strawberries, and its efficiency was demonstrated by participating to interlaboratory European tests where very good Z-scores were obtained.

Keywords: *2,4-D, growth plant stimulator, LC-MS/MS, Miniluke, QuEChERS*

INTRODUCTION

2,4-dichlorophenoxyacetic acid (2,4-D) is the active ingredient in several formulation of herbicides recommended for the control of broadleaf weeds. Other uses include the control of aquatic weeds, some woody vegetation, and site preparation and conifer release in forests. [1]

2,4-D was used as herbicide in the herbicide Agent Orange, a 1:1mixture of 2,4-D and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). Agent Orange was a herbicide widely used during the Vietnam war, and was often contaminated with 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD), which

[a] *1 Decembrie 1918 University, Faculty of Law and Social Sciences, Department of Physical Education and Sports, 15-17 Unirii str., Alba Iulia, Romania*
[b] *Babeş-Bolyai University, Faculty of Chemistry and Chemical Engineering, 11 Arany Janos str., RO-400028, Cluj-Napoca, Romania*
* *Corresponding author: malleda@yahoo.com*

result from the manufacture of 2,4,5-T, and this contaminant has high potential to be carcinogenic, teratogenic, and fetotoxic.[2]

The selective herbicide, 2,4-D, is used to protect grain crops against leafy weeds. It is also applied as a growth stimulator during the growth of plants (tomatoes), and post-harvest for the protection of fruits, especially citrus fruits.

After some experiments on various fruits and vegetables (lichi, pineapple, egg plant), Howell and Wittwer found that a single spray with 2,4-D on tomatoes (100 – 500 mg/L, somewhat dependent on the variety of herbicide) produced prompt flower formation. [3]

Many publications on the physiology of the tomato plant present that application of 2,4-D (2,4-dichlorophenoxyacetic acid), 4-CPA (4 chlorophenoxyacetic acid), and NOA (2-naphthoxyacetic acid) at recommended concentrations will increase fruit size and setting as well as accelerate fruit ripening. Even from 1953, was discovered that application of 2,4-D, NAA (Naphthaleneacetic acid), TIBA (2,3,5-triiodobenzoic acid), NOA (2-naphthoxyacetic acid) and IAA (Indole-3-acetic acid) inhibited Fusarium wilt in tomatoes. So, those plant growth regulators are important not only in increasing crops but also in controlling plant disease. [4, 5]

Today, 2,4-D is also applied as herbicide, a growth plant stimulator (but not approved in our country), and post-harvest protection of fruits, especially citrus fruits.

2,4-D mimics the effect of some natural plant growth regulating hormones (e.g. auxins), and thus stimulates growth, rejuvenates old cells, and overstimulates young cells leading to abnormal growth patterns and death in some plants. 2,4-D resistant plants convert the chemical into inactive, nontoxic carbohydrate conjugates, while susceptible plants convert it into amino acid conjugates which obstruct normal nucleic acid metabolism and protein synthesis. This obstruction affects the activity of enzymes, respiration, and cell division and therefore the plants treated with 2,4-D often exhibit malformed leaves, stems, and roots. [6]

Tomatoes are considered one of the most sensitive crops regarding 2,4-D and its derivatives. As a growth stimulator, it is applied in sublethal doses ranging between 0.42 - 13.44 g s.a /ha directly on the plants, in different stages of growth, from the beginning of flowering. For tomatoes, the tolerance to 2,4-D increases a lot with the age of the plant.

Due to the appearance on the Romanian market of some tomatoes of a abnormally shape (appearance of a tassel), with signs of phytotoxicity due to exposure to overdoses of the herbicide 2,4-D used as a growth regulator, the growth stimulation products based on 2,4-D were withdrawn from market.

Specialists in horticulture support that in our country are very few varieties of tomatoes (e.g. Prekos variety) that naturally produce this varietal character, that tassel, given by a gene, the B gene. Even in this cause, if that tassel is very pronounced, is a clear indication that growth plant stimulators were used, either for pollination (e.g. 2,4-D) or for forced fruit ripening (e.g. Ethrel). [8]

Ethrel is also withdrawn from the romanian market since 2013, but it is still used for various cultures (as well as 2,4-D), the source of supply of such products being the EU countries where they are still approved for their use and non-EU countries.

In our country are approved as growth plant stimulators sodium o-nitrophenolate, sodium 5-nitroguaiacolate and 1-methylcyclopropene - all of these just for fruits protection.

Few growers from all countries, including from us, follow the approved growth regulators and recommended application dosage, but many of them use unapproved products and high concentrations under the impression that a higher dose gave more effective results.

Many studies have already established that for tomato fruits exceeding recommended dosages (e.g. at twice the recommended concentration), produces deformed fruit (reduced fruit quality), increases fruit number and the appearance of a jelly tissue with immature seeds. Also, significant abnormalities with deformed shapes and poor pulp development were observed externally and in transverse sections. [4,5,9]

Other tests indicate that the fruits derived from the plants which were not treated with growth regulators were characterized by the smallest amount of jelly tissue while the fruits set under the influence of 0.001 % 2,4-D + 0.001 % BAP (benzylaminopurine) had the largest jelly tissue amount. The greatest differentiation was found in fertility which ranged from 7.5 seeds from the fruit derived from the plants treated with 0.005 % 2,4-D, to 75.7 seeds from the non-treated plants' fruit. [5,7,9]

In conditions without precipitation, 2,4-D is absorbed by the plant 4-6 hours after herbicide solution application, and if precipitation occurs, 2,4-D dissolves in rainwater and is absorbed by the plant through the vapors on the plant and from the soil.

So, when higher concentrations are used these substances can modify primary plant metabolisms and be unsafe to public health because these plant growth substances have an accumulative and residual effect. These products being synthetic substances, should be used only at recommended concentrations in order to preserve crop quality and not for obtaining large harvests in the shortest possible time, which can affect public health.

RESULTS AND DISCUSSION

In EU the residues of acidic pesticides (as 2,4-D) can be defined as free acids, esters or additionally conjugates. Conjugated residues are formed in crops as secondary products when acids was covalently bonded to different matrix components via ester- , glycoside- and other bonds.

In crop production acidic pesticides are applied either as free acids or esters linked to a variety of alcohol groups. Hydrolysis plays a key role in the mechanism of action of phenoxy-acid derivatives in plants and soil, and because of these reason most esters are reported as free acids. [9]

In analysis of acidic pesticides when residues definition include esters and/or conjugates is necessary to release acids by break-up of any covalent bonds between acidic pesticides and matrix-components, and this involves alkaline hydrolysis, enzymatic hydrolysis or a combination of both.

In 2007 the European Union Reference Laboratories (EURL) distributed on their site a Single Residues Method (SRM) that presented an extraction method of acidic pesticides, including 2,4-D, from wheat flour samples, where alkaline hydrolysis is performed at room temperature before QuEChERS extraction method. A few years ago, Anastassiades et all, presents a study with a new approach of hydrolysis that employs esterase enzymes in order to achieve full hydrolysis of sterically hindered esters, and similar to alkaline hydrolysis, this enzymatic hydrolysis is performed prior to QuEChERS. [10]

In our country , at the Directorate General for Health and Consumer Protection of the European Commission (DG SANCO) recommendation, the necessity to develop a single residues method for 2,4-D analysis appeared, when a problem was found, which actually persists even now, namely that of determining the 2,4-D residues in tomatoes, because there was more and more suspicion at the local level, that the tomatoes in the agro-food markets are treated with a product that gives them an unnatural appearance and are tasteless. The documentation for both the development of the analysis method and the development of the pesticide extraction method from tomatoes constituted a difficult process of finding information, as very few articles and books were found in the specialized literature dealing with this subject, the results were contradictory. [10,11]

Due to its polar nature 2,4-D is difficult to analyze; it is partially linked to the matrix compounds; a good increase in extractability can be achieved by alkaline hydrolysis (e.g. NaOH, K_2CO_3), but even in this situation the recovery rates are up to 65%. A European method (Alkaline hydrolysis preceding QuEChERS for breaking up conjugates (prior to adding acetonitrile)) has been developed for the determination of phenoxyacid pesticides in flour, including 2,4-D, which uses alkaline hydrolysis in the extraction method, whose part of the analysis method has many elements in common with the

one proposed in the present study, the significant difference being in the method of extraction. [10,11].

Also, the analysis method by LC-MS/MS QQQ was a challenge because in matrices of plant origin and animal origin 2,4-D expressed as 2,4-D contain a sum of 2,4-D, its salts, its esters and its conjugates.

In order to establish the most efficient method of extracting 2,4-Dfrom tomatoes, we compared the QuEChERS method and the Miniluke method, with an alkaline hydrolysis previously applied for both.

Both qualitative (chromatographic) and quantitative results were against the QuEChERS method. In figures 1 and 2 were presented the differences between the shape, the amplitude and the areas of the chromatographic peaks obtained by the two extraction methods (HA QuEChERS - alkaline hydrolysis and HA MiniLuke- alkaline hydrolysis), the LCMS analysis method being the same.

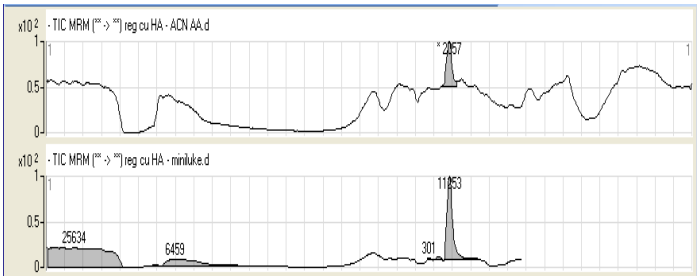

Figure 1. TIC chromatograms for QuEChERS HA - ACN AA) and HA Miniluke extraction method

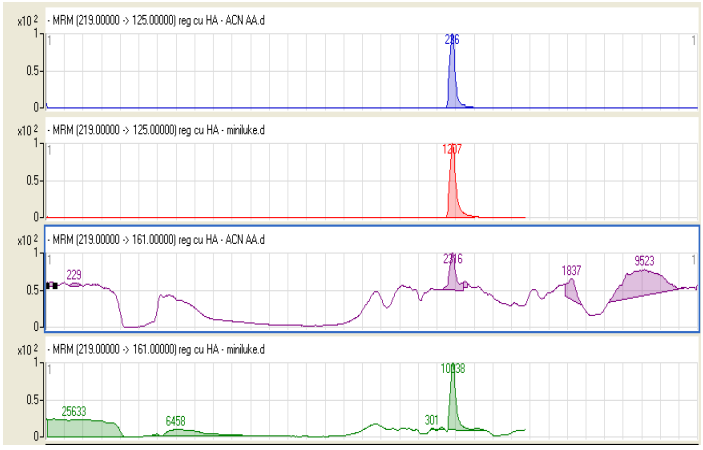

Figure 2. Peak areas for transitions 219→125 and 219→161 with HA Quechers (ACN AA) and HA Miniluke extraction method

The extraction method used by us is a combination of two known methods, namely in the first step an alkaline hydrolysis of the sample is performed, followed,30 minutes after of the neutralization step, by a slightly modified MiniLuke method. This step is performed with the aim of breaking any covalent bonds between acidic pesticides and matrix components.
The extraction procedure is shown schematically the following figure.

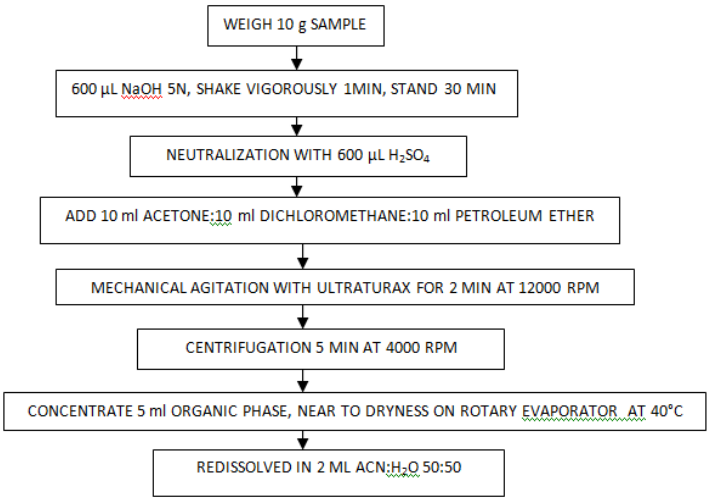

Figure 3. Extraction procedure of 2,4-D from tomatoes

CONCLUSIONS

The analysis method was validated on the matrix of tomatoes (matrix with high water content) and strawberries (matrix with high acidity content), and its efficiency was proven by participating in interlaboratory European tests organized by the European Reference Laboratory for the Analysis of Pesticide Residues from Almeria, Spain where very good Z scores were obtained.

The methods of analysis and extraction of 2,4-D proved effective for matrices with high water content (tomatoes) and those with high acidity (strawberries), but for samples with high starch content (cereals), the extraction method must be modified and adapted, by introducing a certain amount of cold water before the solvent extraction process. The amount of water varies depending on the type of sample (type of cereals) and its granulation after grinding.

The development and validation of 2,4-D extraction method from cereals will be the subject of a subsequent paper.

EXPERIMENTAL SECTION

LC-MS/MS QQQ analysis

For this LC-MS analysis, an AGILENT liquid chromatograph equipped with a quaternary pump model 1200, autosampler and a mass spectrometer triple quadrupole AGILENT 6410A, ionization source type Multi mode ionization (MMI), with electrospray ionization (ESI) in the negative mode.

All the solvents used for the development of the method were of HPLC purity, manufactured by Sigma Aldrich, and the analytical standard 2,4-D was also purchased from Sigma Aldrich. The other reagents were purchased from Merck, and the ultrapure water was produced with the TKA system consisting of two units, Lab Tower and GenPure.

The analytical column was a Zorbax Eclipse XDB-C18, 3.5 microni, 2.1x150mm, (Agilent) kept at 25 °C in the method. Injection volume was 5μL and flow rate of mobile phase was 0.35 ml /min. Mobile phase A was water and phase B was acetonitrile with 0.1% formic acid. Elution gradient was 50% B from the start ramped to the 100% B over the 1 minute, then was ramped at 0% B over the 6 minutes and ramped again at 50% B held until 7 minutes. Energy Fragmentation was established at 70V. Energy Collision for transition 219→161, was established at 10eV, and for transition 219→125 at 25eV. Capillary voltage was setting at 2500V, the temperature of the gas in the ion source at 350 ° C, nebulizer pressure at 60 psi and drying gas flow at 5 L / min. Nitrogen was used as nebulization, desolvation and collision gas.

Method validation

The modified Miniluke extraction method was validated for the tomato matrix, by the LC-MS/MS QQQ method developed in this work, in MRM (Multireaction Monitoring) mode, negative ESI.

Stock solutions of 1000 μg/mL were prepared in acetonitrile and kept in a freezer at -18°C. A 10 μg/mL concentration solution is obtained from the stock solution by dilution with acetonitrile: water 50:50, v/v, this solution being used to determine the optimal fragmentation and collision energies. Another 1μg/mL solution obtained from the 10 μg/mL solution by diluting with acetonitrile: water 50:50, v/v, is used to prepare the calibration standards, this solution if kept cold can be used for several months. The working standards (calibration levels) are obtained by diluting the mixture of pesticides with a concentration of 1 μg/mL, with acetonitrile: water 50:50, v/v, and are prepared whenever needed, being stable for several months if they are kept in optimal conditions (cold and in brown bottles).

In accordance with the guide DG SANCO 12495/2011 and DG SANCO 12571/2013, implemented on 01/01/2014, the following validation criteria are checked for the pesticide 2,4-D in the tomato matrix. [12]

Different types and modes of mass spectrometric detectors provide different degrees of selectivity and specificity, which relates to the confidence in identification. The DG SANCO requirements for LC-MS/MS identification are ≥ 2 product ions. For 2,4-D identification was used 2 transitions 219→161, and 219→125. According DG SANCO documents, the relative intensities or ratios of product ions (MS/MS), expressed as a ratio relative to the most intense (product) ion, should correspond to those of the calibration standard at comparable concentrations and measured under the same conditions. Matrix-matched calibration solutions may need to be used. For LC-MS/MS techniques, recommended maximum tolerances for ion ratio were ± 30%, this criteria has been achieved by proposed analysis method, ion ratio for the two transitions used, being between 8.9 % and 12.1%, depending on the pesticide concentration. (Figure 4)

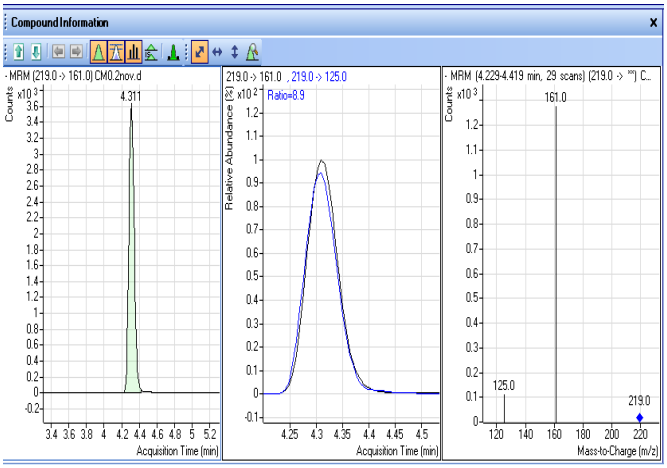

Figure 4. Selectivity and specificity and ion ratio for 2,4-D transitions

Linearity and LOQ

Linearity will be investigated for the pesticides of interest using a 4 point calibration curve (0.025; 0.05; 0.1; 0.2 µg/mL). Calibration levels are prepared in 100 ml volumetric flasks by diluting a standard solution of intermediate concentration of 1 µg/mL with acetonitrile: water 50:50, v/v. We do not work with internal standard.

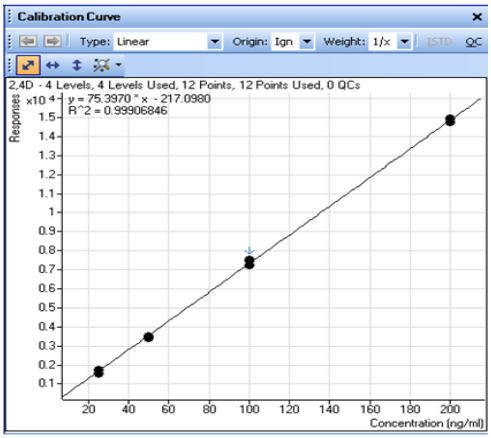

Figure 5. Calibration curbe for 2,4-D in fortified blank matrix

The range of linearity is between 0.025-0.2 mg/kg. The linearity of the calibration curves was evaluated both in the solvent (acetonitrile: water 50:50, v/v) and in a blank matrix fortified at the levels of 0.025µg/mL, 0.05µg/mL, 0.1µg/mL and 0.2µg/mL. Each calibration solution was injected three times (n=3) and the relative standard deviation RSD, mean peak areas, calibration curve equation and correlation coefficient were specified.
The correlation coefficient (r^2) must be greater than or equal to 0.98.

The LOQ was calculated by injecting lower pesticide concentration in spiked tomato extract that yielded a S/N equal to or slightly higher than 10. Also, by definition, LOQ was the lower concentration level of pesticide for which the acceptability criteria were demonstrated: the average recovery rate should be in the range of 70-120%, the standard deviation STDEV ≤ 20%, the relative standard deviation RSD ≤ 20% and the LOQ ≤ MRL.

The LOQ (0.025 mg/kg) was much lower than the MRL's established by the EU legislations for tomatoes (0.05 mg/kg).

Retention time, regression coefficients (r^2), LOQ, recoveries, coefficient of variation RSD, matrix effects and MRL for 2,4-D in tomatoes are presented in the Table 1.

Table 1. Retention time, regression coefficients (r^2), LOQ, recoveries, coefficient of variation RSD (n= 5) %, matrix effects and MRL

R.T (min)	r^2	LOQ	Spiking level 0.05 mg/kg			Spiking level 0.15 mg/kg			MRL mg/kg
			Recovery %	RSD (n=5) %	Matrix effect	Recovery %	RSD (n=5) %	Matrix effect	
4.311	0.999068	0.025	110.5	5.6	- 4.5	95.8	3.29	-5.5	0.05

Matrix effect

Being a rather charged matrix due to the co-extractive compounds, especially lycopene, it was expected to observe a matrix effect.

The matrix effect was calculated on a control matrix that was fortified at 0.05 mg/kg (MRL level for 2,4-D in tomatoes) and 0.15 mg/kg, making an average of five injections from each fortification level, the analyte concentration was analyzed and calculated. (Table1)

The extent of matrix effect can be measured in each analytical sequence by comparing calibration standards of the same concentration in solvent vs. those in matrix extracts.

The more different the slopes of the two curves are, the farther the curves are from each other, respectively the greater the matrix effect. (Figure 6)

The difference in the slopes of the calibration curves can be observed, which does not exceed 50%, so it can be argued that in this matrix the residual values of 2,4-D will not be debatable in the situation where the measurement uncertainty is estimated at ± 50%.

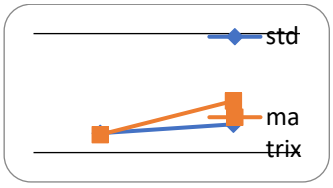

Figure 6. Matrix effect of 2,4-Dcalculated by the solvent calibration curve vs matrix calibration curve

The same conclusion is supported by the matrix effect calculation using a formula:

ME (%)=(conc.of 2,4-D in matrix – conc of 2,4-D in solvent)/conc. of 2,4-D in matrix*100

Recovery and precision

Recovery studies were performed to examine the efficiency of extraction method. Blank samples was spiked at level 0.05 mg/kg and 0.15 mg/kg with five replicates at each level, by standard addition method, and then the recoveries were calculating. The obtained recoveries are shown in Table 1. The average recoveries were 110.5% and 95.8%, indicating that the criteria for this validation parameter was achieved.

Precision was evaluated by analyzing five fortified blank tomato samples for each level at the two concentration levels of the recovery studies. The precision was expressed as the RSD values, and the results is presented in Table 1. The obtained values for this validation parameter was according to DG SANCO criteria's. [12]

Measurement uncertainty

The uncertainty will be calculated taking into account both systematic errors (fidelity) and random errors (repeatability, reproducibility). The expanded uncertainty will be established using a 95% confidence interval.

Table 2. Components of measurement uncertainty

Pesticide	Sum of squares	Ucomb/c	Ucomb	U expand	U%
2.4D	4.6215E-03	6.80E-02	6.93E-03	0.01386	13.86

The measurement uncertainty is below 50%, so according to the DG SANCO 12495/2014 guide, in routine analysis, an uncertainty of ± 50% can be attributed.

REFERENCES

1. J.J. Menn; *Environ. Health Persp*., **1978**, Vol. 27, 113-124
2. D.E. Lilienfeld, M.A. Gallo - *Epidemiologic Reviews*, **1989** - hero.epa.gov
3. M.J. Howell, S.H. Wittwer; *Amer. Soc. Hort. Sci. Proc*., **1954**, *66*, 279-283
4. M. Gemici; B. Turkyilmaz, K. Tan; *JFS,* **2006**, Vol 29, 24-32
5. P. Nowaczyk; L. Nowaczyk; *Acta Physiologiae Plantarum*, **2000**, *22*, 309–311
6. W.R. Mullison; *Environmental fate of phenoxy herbicides*, Biggar, J.W.Seiber, J.N. (eds.).- Oakland (USA): University of California, **1987**, p. 121-131
7. D. Gelmesa; B. Abebie; L. Desalegn; *Sci.Technol.Arts Res*, **2013**, *2*, 25-34
8. C. Vînătoru; B. Mușat; C. Bratu; *Tratat de legumicultură specială,* Editura ALPHA MDN, **2019**
9. M.R. Warmund; M.R. Ellersieck; R.J. Smeda; *Agriculture,* **2022**, *12*, 1489
10. M. Anastassiades, CRL for Single Residue Methods, **2007**
11. EURL-SRM, Analytical Observations Report, *Analysis of Acidic Pesticides Entailing Conjugates and/or Esters in their Residue Definitions*, **2020**, 1
12. DGSANCO/12495/2014, *Method validation and quality control procedures for pesticides residues analysis in food and feed,* **2014**