

Pesticide Residues Method Validation by UPLC-MS/MS for Accreditation Purposes

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The development and validation of an analytical method for the determination of 51 pesticides in tomato using UPLC-MS/MS (ultra performance liquid chromatography coupled to tandem mass spectrometry) was performed in order to fulfill the requirements of the NBR ISO/IEC 17025:2005. The goal of the validation was to evaluate all required parameters, such as linearity of analytical curves, instrument and method limits of detection and quantification, matrix effect and accuracy (trueness and precision). Stock solutions of the pesticides were prepared in organic solvent by two different analysts independently, in order to validate this preparation by comparing the detector response areas of injections ($n = 7$). Pesticides were extracted from tomatoes applying the Dutch mini-Luke method with 30 mL of each solvent as acetone, petroleum ether as well as dichloromethane. A small aliquot of the organic solvent extract was evaporated and reconstituted in methanol with 0.1% of acetic acid (v/v) for UPLC-MS/MS analysis. The recovery experiments were done by spiking blank samples at 10, 20 and 50 $\mu\text{g kg}^{-1}$, performed by two analysts, seven replicates for each concentration level plus the blank sample. From the 51 studied compounds, 46 showed recoveries within the acceptable range of 70-120% for all evaluated concentrations. For 82% of pesticides, the validated method limit of quantification was the lowest concentration studied (10 $\mu\text{g kg}^{-1}$). The intermediate precision was below 20% for all evaluated pesticides showing excellent repeatability of the method.

Keywords: validation, accreditation, pesticides, Dutch mini-Luke method, UPLC-MS/MS

Introduction

Since 2008, Brazil has had the worrying position of being the largest consumer of pesticides in the world. According to the Ministry of Agriculture, Livestock and Supply (MAPA), in that year 673.9 t of these toxic products ended up in the environment.¹

The extensive use of pesticides is a serious public health problem in developing countries, especially those with economies based on agribusiness, as is the case in Brazil. Two monitoring programs for pesticide residues are currently in place in Brazil aiming to evaluate compliance with national maximum residues levels (MRL). The Program on Pesticide Residues Analysis in Food (PARA), coordinated by the National Health Surveillance Agency (ANVISA), aims to analyze fruits and vegetables sold

at Brazilian market places. The National Residue and Contamination Control Program (PNCRC), coordinated by MAPA, intends to control animal products, as well as fruits and vegetables products that are destined mainly for exportation.²

Furthermore, there is a need to develop rapid and accurate analytical methods for determination of pesticide residues in agricultural products, including a wide range of pesticide groups, with very different properties (polar, non-polar, planar, acids, bases), and doing that in a single chromatographic run and one extraction *per* sample.^{3,4}

Thus, considering the increase in the use of pesticides in various cultures, there is a growing need for government agencies to carry out control programs to determine the occurrence of residues and their concentrations. The main difficulty in developing reliable methods is the lack of enough laboratories in Brazil with competence on identification and quantification of these substances.

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Laboratory accreditation appears in this context as a powerful tool to demonstrate the ability of laboratories not only in the field of determining pesticides residues in foods, but in all areas where the presence of a quality management system is required to act accurately and effectively in order to demonstrate the ability of laboratories to solve problems that occur.

Laboratory accreditation is a way to determine the technical competence of laboratories to perform specific types of tests, measurements and calibration. It also provides formal recognition of competent laboratories, thus providing an easy way for customers to identify and select reliable testing, measurement and calibration services able to meet their needs.⁵

Many countries around the world have one or more organizations responsible for laboratory accreditation. Most of these organizations adopt the ISO/IEC 17025 norm⁶ as the basis for accrediting testing and calibration laboratories.⁷

The General Accreditation Coordinator (CGCRE) linked to the National Institute of Metrology, Standardization and Industrial Quality (INMETRO) is the accreditation body for conformity assessment recognized by the Brazilian government, as laid down in decree No. 6275 of November 28, 2007. Therefore, CGCRE is, within the organizational structure of INMETRO, the main organizational unit that has full responsibility and authority over all aspects of the accreditation, including accreditation decisions.⁷

Laboratory accreditation by CGCRE is performed by the Laboratory Accreditation Division (DICLA), which performs activities related to the granting and maintenance of accreditation, in accordance with requirements of the NBR ISO/IEC 17025 norm.⁸

Accreditation involves a complete review of all structural parts of the laboratory that contribute to generate accurate and reliable results. The criteria that the auditors refer for quality control are based on an international standard (ISO/IEC 17025:2005), which is applicable to all laboratories involved in sampling, development of new methods, testing and calibration. Official accreditation bodies use this international standard, published by the International Organization for Standardization (ISO), to ensure the parameters that influence the laboratory's ability to generate precise and accurate results of tests and/or calibration, including adequate control of the procedures, quality, qualification, training and experience of all personnel involved, as well as proper maintenance and calibration of all equipment.⁹

Efficient management of the quality system enhances and improves development, competence and discipline of

the staff. The accreditation allows the laboratory, above all, to assess its performance using it as reference to demonstrate their technical competence through regular reviews.

To be accredited by an accreditation body, it is essential that the laboratory implement a Quality Management System, which is a set of minimum rules in order to guide each part of the organization to run its duties properly, in time and planning, and everything must be directed towards the common goal of the organization: customer satisfaction.

Thus, the objective of this study was to validate an analytical method for the simultaneous determination of 51 pesticide residues in tomatoes, in order to evaluate the method performance and the compliance of the laboratory according with ISO/IEC 17025 requirements.⁶ The validation for tomato as matrix is representative for all crops classified in the same commodity group, which is high water content matrices.¹⁰

Experimental

Reference standards, chemicals and reagents

Methanol, acetonitrile, toluene, acetone, petroleum ether, dichloromethane, acetic acid, all pesticide purity grade were purchased from J. T. Baker Chemical or Mallinckrodt (Phillipsburg and Center Valley, USA respectively). Pesticides reference standards were obtained from Dr. Ehrenstorfer (Augsburg, Germany). Sodium sulfate was obtained from UCT (Bristol, USA) and ammonium formate from Sigma-Aldrich (Seelze, Germany).

Preparation of analytical standard solutions

Stock solutions of individual pesticide reference standards were prepared at 1000 mg L⁻¹ in organic solvent taking into account the solubility and purity of the standard. Reference standards were weighed directly into a 20 mL flask and the required volume of the appropriate solvent (preferably toluene or methanol and only if strictly necessary acetonitrile or acetone) was added using automated and calibrated pipettes. Finally, the solutions were placed for about 5 min in an ultrasonic bath in order to assure complete dissolution.

After the preparation of individual stock solutions, a stock mixture standard solution containing all 51 compounds was prepared at 1 mg L⁻¹ in methanol containing 0.1% of acetic acid (v/v). Therefore, 200 µL of each individual solution was transferred to a volumetric flask of 200 mL and the volume was made up with methanol with 0.1% of acetic acid (v/v). Then, the solution was mixed and homogenized and divided

over four different flasks in order to avoid contamination during handling. This solution was used to prepare all analytical standard solutions in organic solvent and in matrix blank extract for the linearity study of analytical curves.

Analytical solutions were stored in a freezer at 20 °C. Before use, all solutions were allowed to stand until room temperature was reached. Subsequently, the solutions were placed in an ultrasonic bath for 5 min.

In order to validate the stock solutions, they were prepared by two different analysts and their results were compared by UPLC-MS/MS (ultra performance liquid chromatography coupled to tandem mass spectrometry) measurement. The difference in detector response (peak area) of both solutions was calculated according to equation 1.

$$\text{Difference between solution (\%)} = \left(\frac{\text{mean peak area (n = 7) from solution prepared by analyst 1}}{\text{mean peak area (n = 7) from solution prepared by analyst 2}} - 1 \right) \times 100 \quad (1)$$

The criterion for the acceptable difference is < 10%, according to the SANCO document.¹⁰

UPLC-MS/MS experimental conditions

The UPLC-MS/MS system consisted of an ACQUITY UPLC coupled to a XEVO TQ-S (triple quadrupole) tandem mass spectrometer (Waters, Milford, USA).

Chromatographic separations were carried out using a BEH C18 column (2.1 mm × 100 mm, 1.7 µm particle size), maintained at 60 °C with a total run time of 14 min. The mobile phases consisted of 0.3 g L⁻¹ ammonium formate solution (solvent A) and methanol (solvent B). The following gradient elution (0.45 mL min⁻¹ flow rate) was performed: 80% A at the injection time, decreasing linearly to 15% A over 11 min. This eluent composition was maintained for 1 min and then increased linearly to 80% A over 0.5 min. This condition was maintained until the end of the chromatographic run.

The mass spectrometer was operated in the positive electrospray ionization mode, ESI (+). The capillary voltage was settled at 3.00 kV. Desolvation gas temperature was 400 °C. Desolvation gas flow and cone gas flow were 500 and 150 L h⁻¹, respectively. Collision gas flow was used at 0.15 mL min⁻¹. Two MRM (multiple reaction monitoring) transitions *per* analyte were monitored as can be seen in Table 1. One product ion was used for quantification and the ion ratio of both product ions was used for identification, with a ± 30% tolerance window (Table 1).¹⁰

Preliminary tuning experiments were carried out via direct infusion of individual diluted standard solutions (200 ng mL⁻¹) into the mass spectrometer, in order to establish the optimal detection conditions. The infusion flow rate was set at 5 µL min⁻¹.

Table 1. Acquisition parameters for the pesticides analyzed by UPLC-MS/MS

Pesticide	Precursor ion (<i>m/z</i>)	Retention time / min	Cone voltage / V	Quantification transition		Confirmation transition		Ion ratio 1st/2nd
				Product ion (<i>m/z</i>)	Collision energy / eV	Product ion (<i>m/z</i>)	Collision energy / eV	
Acephate	183.9	0.81	20	142.8	10	94.6	25	19.0
Azoxystrobin	404.1	7.10	15	372.0	16	328.9	30	3.4
Bitertanol	338.1	9.25	30	98.9	16	70.1	8	1.0
Buprofezin	306.1	10.29	20	201.0	12	115.9	16	5.4
Carbaryl	219.0	5.01	30	144.9	28	126.9	22	0.3
Chlorpyrifos	350.1	10.59	25	97.0	33	197.9	19	1.1
Chlotianidin	250.0	2.01	20	168.9	14	131.8	14	1.9
Cyproconazole	292.1	7.64	25	70.1	18	124.9	30	4.4
Difenoconazole	406.1	9.66	35	250.9	25	187.8	40	7.4
Dimethoate	230.0	2.30	20	198.8	10	124.8	22	2.8
Dimetomorph	388.1	7.40	35	300.9	20	165.0	30	2.2
Diuron	233.0	5.80	25	72.0	18	159.9	25	14.3
Etofenprox	394.3	12.03	20	177.0	15	106.9	43	5.3
Fenarimol	331.0	8.08	40	81.0	30	268.0	25	1.0
Fenthion	279.0	8.78	25	168.9	18	104.9	25	1.9
Fludioxonil	266.0	7.16	15	158.0	28	229.0	10	1.5
Flufenoxuron	489.0	10.96	30	158.0	20	141.0	40	1.5
Flutriafol	302.1	6.02	25	70.1	16	122.9	30	4.5
Hexaconazole	314.1	9.07	30	70.1	20	158.8	40	9.0
Imidacloprid	256.1	2.00	25	174.9	20	209.0	12	1.2
Indoxacarb	528.1	9.75	30	202.9	40	217.9	25	1.2
Kresoxim-methyl	314.2	8.73	30	115.9	12	131.0	25	0.7

Table 1. Acquisition parameters for the pesticides analyzed by UPLC-MS/MS (cont.)

Pesticide	Precursor ion (<i>m/z</i>)	Retention time / min	Cone voltage / V	Quantification transition		Confirmation transition		Ion ratio 1st/2nd
				Product ion (<i>m/z</i>)	Collision energy / eV	Product ion (<i>m/z</i>)	Collision energy / eV	
Linuron	249.0	6.70	20	159.9	20	181.9	16	1.3
Lufenuron	511.0	10.68	25	158.0	15	141.0	40	1.4
Malathion	331.0	7.40	30	126.9	12	98.9	25	2.0
Methamidophos	141.9	0.72	30	93.9	12	124.8	14	2.6
Methidathion	303.0	6.11	30	84.9	20	144.8	10	1.5
Methomyl	162.9	1.34	15	88.0	10	105.9	10	0.7
Methoxyfenoside	369.2	7.71	30	148.9	18	313.0	8	1.6
Omethoate	214.0	0.88	25	124.8	22	182.8	10	0.8
Oxadixyl	279.1	3.95	20	219.0	12	132.3	25	6.4
Oxamyl	237.0	1.10	15	72.0	10	90.0	10	2.7
Pencycuron	329.1	9.51	30	124.9	30	218.0	16	5.3
Pirimiphos-methyl	306.1	9.14	30	107.9	30	67.1	40	1.4
Prochloraz	376.0	9.23	20	307.9	12	70.1	25	2.5
Profenofos	372.9	10.11	30	302.6	20	127.9	40	4.5
Propiconazole	342.1	8.93	35	158.9	20	69.1	30	1.0
Propoxur (IS) ^a	210.1	4.57	15	110.9	12	92.9	25	2.5
Pyraclostrobin	388.1	9.11	25	163.0	25	193.9	12	0.7
Pyriproxyfen	322.2	10.34	25	95.9	15	184.9	23	2.4
Spinosad	746.5	11.63	50	142.0	31	98.1	59	6.3
Tebuconazole	308.2	8.76	30	70.1	24	124.9	40	14.9
Teflubenzuron	381.0	10.24	25	158.0	15	141.0	30	1.2
Thiabendazole	202.0	3.48	45	174.9	25	130.9	30	2.3
Thiacloprid	253.0	3.01	35	125.8	20	90.0	40	4.9
Tolylfluanid	364.0	8.77	15	136.9	30	237.8	14	5.0
Triadimefon	294.1	7.69	30	69.1	20	196.9	16	1.1
Triadimenol	296.1	7.93	30	70.0	10	98.9	15	9.6
Trichlorfon	256.9	2.39	25	108.8	20	126.8	18	10.0
Trifloxystrobin	409.2	9.87	25	185.9	14	145.0	40	1.9
Triflumizole	346.1	9.97	15	278.0	10	73.1	18	4.9
Zoxamide	336.0	8.80	30	186.9	25	158.9	40	2.4

^aIS = internal standard.

Extraction procedure

The method used for pesticides extraction from tomato was the acetone-based Dutch mini-Luke method issued by the Netherlands Food and Consumer Product Safety Authority.^{11,12} The Dutch mini-Luke method is a miniaturized and optimized adaptation of the original Luke method developed in 1975.¹³ The miniaturization enabled a considerable reduction of sample amount and solvents used, as well as analysis time.

Tomato samples were homogenized after stalk removal and 15 g ($\pm 1\%$) were weighed into 250 mL Teflon[®] tubes. Then, 30 mL of acetone was added and the mixture was homogenized with an ultraturrax for 30 s at 15,000 rpm. Sodium sulfate (15 g) was added and mixed with the ultraturrax for 30 s. After this step, 30 mL of petroleum ether and 30 mL of dichloromethane were added and

mixed (ultraturrax) for 30 s to induce partitioning. The tubes were centrifuged for 3 min at 20 °C and 3600 rpm. After centrifugation, 1.2 mL of extract was transferred to evaporation tubes and the organic solvent mixture was evaporated (stepwise first at 45 °C, then at 65 °C) until nearly dry. Complete dryness was achieved in a fume hood, letting the tubes stand at room temperature. Finally, 1 mL of methanol with 0.1% of acetic acid (v/v) and the internal standard (propoxur at 40 ng mL⁻¹) was added to reconstitute the extract using a vortex mixer. Figure 1 shows the extraction procedure.

Method validation

All the validation parameters were evaluated following the orientation document DOQ-CGCRE-008 (Orientation of Analytical Methods Validation) provided by INMETRO.¹⁴

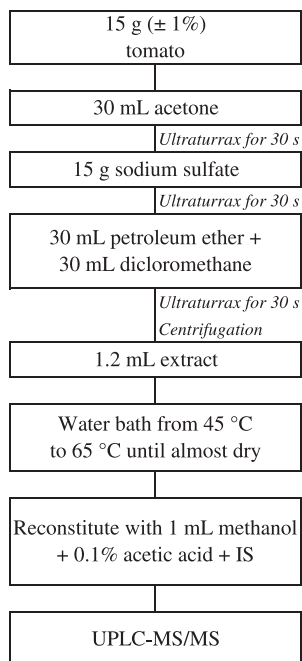


Figure 1. Scheme of the Dutch mini-Luke extraction method used for pesticides residue method validation. IS = propoxur at 40 ng mL⁻¹.

Calibration curves, linearity, limit of detection (LOD) and limit of quantification (LOQ)

Linearity of analytical curves was evaluated by injecting analytical solutions prepared in solvent (methanol with 0.1% of acetic acid, v/v) and in blank tomato extracts at 0.1, 0.2, 0.5, 1.0, 5.0, 20.0 and 100.0 ng mL⁻¹.

First, the blank of solvents and reagents (extraction procedure without the presence of matrix and analytes) and matrix blank extract (matrix extraction without analytes) were injected. Then, the analytical solutions prepared in organic solvent and in blank matrix extract were injected. These solutions were injected in ascending concentration order to avoid carry over in the chromatographic system between injections. Furthermore, solutions in solvent and in blank matrix extract were injected alternately. This sequence was repeated seven times.

Using data obtained from the linearity study for each analyzed pesticide, the estimated instrument limit of detection (LOD_i) and method (LOD_m), as well as estimated instrument limit of quantification (LOQ_i) and method (LOQ_m) were calculated. The estimated LOQ_m was evaluated against the target validated LOQ_m (10 µg kg⁻¹).

Matrix effect evaluation

Components present in tomatoes (typically of natural origin) can influence the quantitative measurement of pesticides without being detected as interfering compounds. Some analytes are more affected than others.^{15,16}

The matrix effect is the effect observed by the increase or decrease in detector response for a particular analyte present in the matrix extract compared to the detector response for the analyte in organic solvent.¹⁷

In this study, the matrix effect was evaluated in two different ways. The first way was by comparison of peak areas obtained from analytical solutions prepared in organic solvent and from those in blank matrix extract at each individual concentration, according to equation 2.

$$\text{Matrix effect (\%)} = \left[\frac{\left(\frac{\text{peak area std in matrix}}{\text{peak area std in solvent}} \right) - \left(\frac{\text{peak area std in matrix}}{\text{peak area std in solvent}} \right)}{\text{peak area std in solvent}} \right] \times 100 \quad (2)$$

The second way was performed by comparing the slopes of the analytical curves obtained from solutions prepared in organic solvent and those in the blank matrix extract, and calculated via equation 3.

$$\text{Matrix effect (\%)} = \left[\frac{\left(\frac{\text{slope analytical curve standard in matrix}}{\text{slope analytical curve standard in solvent}} \right) - \left(\frac{\text{slope analytical curve standard in matrix}}{\text{slope analytical curve standard in solvent}} \right)}{\text{slope analytical curve standard in solvent}} \right] \times 100 \quad (3)$$

Using those two methods, it was possible to determine whether the matrix caused a positive (increase of the detector response) or negative effect (decrease of the detector response).

Accuracy and precision

In order to evaluate the accuracy (trueness) and precision of the method, recovery experiments were done. Blank tomatoes were spiked with a known amount of the mixture solution (1 mg L⁻¹) to obtain a concentration of all analytes studied at 10, 20 and 50 µg kg⁻¹. For each concentration, seven replicates were performed. Seven replicates of the blank sample extract were also prepared. Accuracy in terms of recovery percentage was calculated according to equation 4.

$$\text{Recovery (\%)} = \left[\frac{\left(\frac{\text{analyte concentration determined in spiked sample}}{\text{analyte concentration added to spiked sample}} \right) - \left(\frac{\text{analyte concentration in blank sample}}{\text{analyte concentration added to spiked sample}} \right)}{\text{analyte concentration added to spiked sample}} \right] \times 100 \quad (4)$$

To evaluate the repeatability of the analytical method in terms of intermediate precision, the fortification and recovery tests were performed by two different analysts on different days. The trueness, expressed as average percentage recovery of seven replicates for each concentration was calculated for each pesticide studied.

Thereafter, the average of averages from the results of both analysts and the standard deviation for each concentration were calculated. From these results, the precision, expressed as RSD (relative standard deviation), was calculated for each level for each pesticide.

Results and Discussion

Selected internal standard

An internal standard is a chemical substance added to a known matrix portion or matrix extract in a specific step of the analysis in order to assess the correct execution of the test procedure (or part of it). The internal standard must be chemically stable and must preferably demonstrate similar behavior as of the target analytes.¹⁰

In pesticide residue analysis, the internal standard generally is a pesticide that has its use banned or a compound that is not found in the matrices. For this study, the pesticide propoxur (at 40 ng mL⁻¹) was used, which was also added to all analytical solutions. For the analytical curves as well as in the samples used to evaluate the accuracy and precision of the extraction method. This compound had the purpose of identifying potential losses and errors during the whole procedure.

Despite the use of propoxur as QC (quality control) internal standard, quantification was done via external calibration.

Validation of stock solutions

The preparation of stock solutions was considered satisfactory if the difference between the results from both analysts was less than $\pm 10\%$.¹⁰

For 43 of the 51 studied compounds, the difference (%) between areas of the same substance obtained from analysis of solutions prepared by two different analysts, were between -7.6 and 5.6% . These results show the excellent preparation procedure of stock solutions.

Method validation

Analytical curves and linearity

To evaluate the linearity of analytical curves, the solutions were prepared in organic solvent (methanol with 0.1% of acetic acid, v/v) and in blank tomato extract.

The determination coefficient (r^2) value numerically expresses the percentage of total analytical signal variation due to the analyte concentration variation. This percentage varies from 0 to 100% and r^2 value ranges from 0 to 1. Ideally, the closer the percentage of the variance observed in the analytical signal by varying the concentration is to 100%, the closer the r^2 value will be to 1. Consequently, the quality of the calibration curve will be better, because the variation of the analytical signal as a function of analyte concentration will be more sensitive and best represented by the linear equation obtained.

As can be seen in Table 2 from the 51 studied compounds, both in organic solvent and in matrix extract, 47 (92%) showed a determination coefficient greater than or equal to 0.99. On the other hand, bitertanol, carbaryl, lufenuron and teflubenzuron showed r^2 between 0.96 and 0.99 for both analytical curves. These results are in accordance to INMETRO requirements.¹⁴

LOD and LOQ

Table 3 shows the limits of detection and quantification (of the instrument and method) calculated from individual solutions prepared in methanol with 0.1% of acetic acid (v/v) and in blank matrix extract.

For analytical solutions prepared in organic solvent, 28 of the 51 studied pesticides showed a LOD_i of 0.1 ng mL⁻¹. For 10, 4 and 6 compounds, the calculated LOD_i was 0.2, 0.5 and 1.0 ng mL⁻¹, respectively.

For analytical solutions prepared in blank matrix extract, 25 of the studied pesticides showed a LOD_i of 0.1 ng mL⁻¹. For the other 10, 8 and 3 compounds, the calculated LOD_i was 0.2, 0.5 and 1.0 ng mL⁻¹, respectively.

As can be seen in the Table 3 is possible to note that for some compounds the limits are much different when results in solvent are compared to blank matrix extract. For carbaryl for example, the LOD_i when solutions were prepared in blank matrix extract is 50 times greater compared to results when solutions were prepared in organic solvent.

Matrix effect

The matrix effect is the effect observed by the increase or decrease in detector response for a particular analyte present in the matrix extract compared to the detector response for the analyte in organic solvent.¹⁸

For LC-MS/MS (liquid chromatography coupled to tandem mass spectrometry) analysis this effect is usually caused by interference of matrix components eluting near or at the same retention time of the analyte and competing with it during the ionization process. At low concentrations, the matrix effect can become significantly important, because there is a reduction in ionization due to lower analyte concentration in matrix.¹⁸

The matrix effect results were evaluated at each individual concentration of the analytical curves according to equation 2 at matrix effect evaluation section, as well as via analytical curves slope of standards in organic solvent and in matrix extract according to equation 3 (Table 3 and Figure 2, respectively).

As can be seen in Table 4, many pesticides showed high matrix effect, or could not be determined, in the four lowest concentrations of analytical curves. This is

Table 2. Linearity data of the target analytes: equation curve and determination coefficient (r^2) of analytical curves obtained from solutions prepared in organic solvent (methanol/0.1% acetic acid) and in blank matrix extract

Pesticide	Organic solvent		Blank matrix extract	
	Equation curve	r^2	Equation curve	r^2
Acephate	$y = 350x - 127$	0.998	$y = 363x - 189$	0.996
Azoxystrobin	$y = 17755x - 606$	0.997	$y = 17142x + 2705$	0.996
Bitertanol	$y = 89x - 142$	0.970	$y = 99x - 318$	0.974
Buprofezin	$y = 12364x + 1244$	0.995	$y = 12202x + 1459$	0.998
Carbaryl	$y = 15x + 35$	0.967	$y = 15x + 65$	0.967
Chlorpyrifos	$y = 4391x + 194$	0.992	$y = 4531x - 14$	0.997
Chlotianidin	$y = 838x + 39$	0.997	$y = 828x + 55$	0.996
Cyproconazole	$y = 2353x + 68$	0.991	$y = 2424x + 75$	0.998
Difenoconazole	$y = 8337x - 1219$	0.998	$y = 8152x + 227$	0.996
Dimethoate	$y = 3416x - 235$	0.999	$y = 3454x - 114$	0.999
Dimetomorph	$y = 2197x - 175$	0.996	$y = 2567x + 238$	0.995
Diuron	$y = 3429x - 405$	0.998	$y = 3403x - 123$	0.999
Etofenprox	$y = 6574x + 118$	0.997	$y = 6588x + 577$	0.998
Fenarimol	$y = 636x - 151$	0.993	$y = 629x + 50$	0.998
Fenthion	$y = 5135x + 413$	0.995	$y = 5215x + 90$	0.991
Fludioxonil	$y = 205x - 23$	0.997	$y = 203x + 16$	0.993
Flufenoxuron	$y = 4625x - 408$	0.996	$y = 4702x - 278$	0.993
Flutriafol	$y = 5527x + 957$	0.997	$y = 5530x + 935$	0.996
Hexaconazole	$y = 3770x - 137$	0.994	$y = 3778x - 83$	0.997
Imidacloprid	$y = 1047x - 69$	0.996	$y = 1054x + 255$	0.996
Indoxacarb	$y = 1207x - 66$	0.993	$y = 1238x - 317$	0.990
Kresoxim-methyl	$y = 96x - 51$	0.998	$y = 93x - 63$	0.993
Linuron	$y = 3621x - 205$	0.998	$y = 3552x + 121$	0.999
Lufenuron	$y = 767x - 134$	0.981	$y = 813x - 288$	0.986
Malathion	$y = 3919x + 246$	0.997	$y = 3919x + 406$	0.998
Methamidophos	$y = 2718x - 350$	0.998	$y = 2662x - 167$	0.999
Methidathion	$y = 526x - 80$	0.997	$y = 518x - 46$	0.997
Methomyl	$y = 62x - 30$	0.996	$y = 62x - 33$	0.995
Methoxyfenoside	$y = 1485x - 80$	0.998	$y = 1476x - 145$	0.996
Omethoate	$y = 2423x - 372$	0.999	$y = 2367x - 65$	0.999
Oxadixyl	$y = 3668x - 532$	0.998	$y = 3643x - 358$	0.999
Oxamyl	$y = 274x - 53$	0.998	$y = 276x - 75$	0.998
Pencycuron	$y = 19571x - 1144$	0.998	$y = 19155x + 1412$	0.998
Pirimiphos-methyl	$y = 10334x + 108$	0.997	$y = 10104x + 1366$	0.996
Prochloraz	$y = 4604x + 307$	0.995	$y = 4774x - 604$	0.998
Profenofos	$y = 5524x - 578$	0.997	$y = 5532x - 431$	0.996
Propiconazole	$y = 3678x - 300$	0.996	$y = 3647x + 631$	0.996
Pyraclostrobin	$y = 7336x - 32$	0.996	$y = 7068x + 1096$	0.997
Pyriproxyfen	$y = 22568x + 3407$	0.998	$y = 22755x + 3395$	0.999
Spinosad	$y = 4366x - 426$	0.996	$y = 4295x - 321$	0.998
Tebuconazole	$y = 5484x + 127$	0.998	$y = 5284x + 1302$	0.993
Teflubenzuron	$y = 847x - 220$	0.984	$y = 752x + 323$	0.978
Thiabendazole	$y = 7610x - 546$	0.999	$y = 7514x + 117$	0.999
Thiacloprid	$y = 10848x + 169$	0.999	$y = 10858x + 951$	0.999
Tolyfluanid	$y = 1153x - 270$	0.997	$y = 1081x + 189$	0.993
Triadimefon	$y = 3898x - 83$	0.997	$y = 3719x + 679$	0.997
Triadimenol	$y = 308x + 10$	0.994	$y = 301x - 20$	0.997
Trichlorfon	$y = 2457x - 566$	0.997	$y = 2468x - 552$	0.998
Trifloxystrobin	$y = 12861x - 199$	0.997	$y = 12747x - 740$	0.992
Triflumizole	$y = 6984x - 948$	0.997	$y = 6829x + 1323$	0.995
Zoxamide	$y = 9190x + 304$	0.996	$y = 9131x + 439$	0.997

Table 3. Estimated instrument and method LOD and LOQ, calculated from the data obtained from analytical solutions prepared in solvent (methanol with 0.1% of acetic acid, v/v) and in blank matrix extract

Pesticide	Methanol with 0.1% of acetic acid (v/v)				Blank matrix extract			
	LOD _i ^a / (ng mL ⁻¹)	LOD _m ^b / (µg kg ⁻¹)	LOQ _i ^c / (ng mL ⁻¹)	LOQ _m ^d / (µg kg ⁻¹)	LOD _i ^a / (ng mL ⁻¹)	LOD _m ^b / (µg kg ⁻¹)	LOQ _i ^c / (ng mL ⁻¹)	LOQ _m ^d / (µg kg ⁻¹)
Acephate	1.0	5.0	3	20	1.0	5.0	3	20
Azoxystrobin	0.1	0.5	0.3	2	0.1	0.5	0.3	2
Bitertanol	5.0	25.0	20	85	5.0	25.0	20	85
Buprofezin	0.1	0.5	0.3	2	0.1	0.5	0.3	2
Carbaryl	0.1	0.5	0.3	2	5.0	25.0	20	85
Chlorpyrifos	0.1	0.5	0.3	2	0.1	0.5	0.3	2
Chlotianidin	0.1	0.5	0.3	2	0.1	0.5	0.3	2
Cyproconazole	0.1	0.5	0.3	2	0.1	0.5	0.3	2
Difenoconazole	0.2	1.0	0.7	5	0.1	0.5	0.3	2
Dimethoate	0.1	0.5	0.3	2	0.1	0.5	0.3	2
Dimetomorph	0.1	0.5	0.3	2	0.1	0.5	0.3	2
Diuron	0.2	1.0	0.7	5	0.2	1.0	0.7	5
Etofenprox	0.1	0.5	0.3	2	0.1	0.5	0.3	2
Fenarimol	1.0	5.0	3	20	1.0	5.0	3	20
Fenthion	0.1	0.5	0.3	2	0.1	0.5	0.3	2
Fludioxonil	1.0	5.0	3	20	0.5	2.5	2	10
Flufenoxuron	0.1	0.5	0.3	2	0.1	0.5	0.3	2
Flutriafol	0.1	0.5	0.3	2	0.1	0.5	0.3	2
Hexaconazole	0.2	1.0	0.7	5	0.2	1.0	0.7	5
Imidacloprid	0.2	1.0	0.7	5	0.5	2.5	2	10
Indoxacarb	1.0	5.0	3	20	0.2	1.0	0.7	5
Kresoxim-methyl	5.0	25.0	20	85	5.0	25.0	20	85
Linuron	0.1	0.5	0.3	2	0.1	0.5	0.3	2
Lufenuron	0.5	2.5	2	10	0.2	1.0	0.7	5
Malathion	0.1	0.5	0.3	2	0.1	0.5	0.3	2
Methamidophos	0.1	0.5	0.3	2	0.1	0.5	0.3	2
Methidathion	0.2	1.0	0.7	5	0.2	1.0	0.7	5
Methomyl	1.0	5.0	3	20	5.0	25.0	20	85
Methoxyfenoside	0.1	0.5	0.3	2	0.2	1.0	0.7	5
Omethoate	0.1	0.5	0.3	2	0.1	0.5	0.3	2
Oxadixyl	0.5	2.5	2	10	0.5	2.5	2	10
Oxamyl	0.5	2.5	2	10	0.5	2.5	2	10
Pencycuron	0.1	0.5	0.3	2	0.1	0.5	0.3	2
Pirimiphos-methyl	0.1	0.5	0.3	2	0.1	0.5	0.3	2
Prochloraz	0.2	1.0	0.7	5	0.5	2.5	2	10
Profenofos	0.2	1.0	0.7	5	0.5	2.5	2	10
Propiconazole	0.5	2.5	2	10	0.5	2.5	2	10
Pyraclostrobin	0.1	0.5	0.3	2	0.1	0.5	0.3	2
Pyriproxyfen	0.1	0.5	0.3	2	0.1	0.5	0.3	2
Spinosad	0.1	0.5	0.3	2	0.1	0.5	0.3	2
Tebuconazole	0.1	0.5	0.3	2	0.2	1.0	0.7	5
Teflubenzuron	0.2	1.0	0.7	5	0.2	1.0	0.7	5
Thiabendazole	0.1	0.5	0.3	2	0.1	0.5	0.3	2
Thiacloprid	0.1	0.5	0.3	2	0.1	0.5	0.3	2
Tolyfluanid	0.2	1.0	0.7	5	0.2	1.0	0.7	5
Triadimefon	0.1	0.5	0.3	2	0.1	0.5	0.3	2
Triadimenol	1.0	5.0	3	20	1.0	5.0	3	20
Trichlorfon	5.0	25.0	20	85	5.0	25.0	20	85
Trifloxystrobin	0.1	0.5	0.3	2	0.2	1.0	0.7	5
Triflumizole	0.2	1.0	0.7	5	0.5	2.5	2	10
Zoxamide	0.1	0.5	0.3	2	0.1	0.5	0.3	2

^aLOD_i = 3 × RSD × concentration; ^bLOD_m = LOD_i × dilution factor (5); ^cLOQ_i = LOD_i × 3.33 (rounded values); ^dLOQ_m = LOQ_i × dilution factor (5) (rounded values).

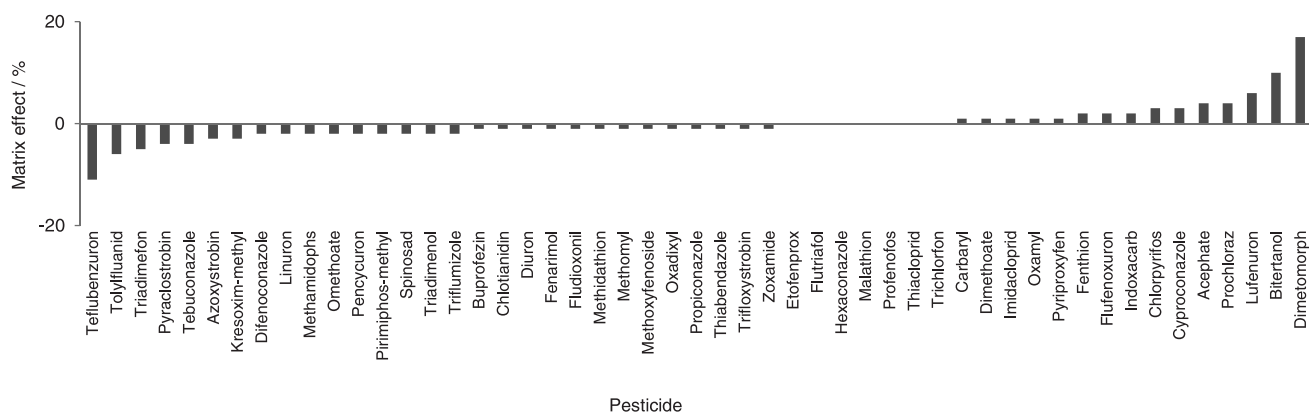


Figure 2. Matrix effect calculated from slopes differences of analytical curves obtained from solutions prepared in organic solvent and those prepared in blank matrix extract.

explained by the fact that in these low concentrations (0.1 to 1.0 ng mL⁻¹), the pesticide amount is reduced when compared to the higher concentrations of matrix compounds and, consequently, are most affected by the presence of these components.

On the other hand, all the compounds evaluated in this

study demonstrated a matrix effect between -20 and +20% when the calculation took only the slope of analytical curves into account, as can be seen in Figure 2. Thus, it is possible to conclude that the tomato matrix does not exert a significant effect on the detector signal from any of the analyzed compounds.

Table 4. Matrix effect calculated for each pesticide by injection of analytical solutions prepared in methanol (n = 7) and in blank matrix extract (n = 7)

Pesticide	Matrix effect / %						
	0.1 (ng mL ⁻¹)	0.2 (ng mL ⁻¹)	0.5 (ng mL ⁻¹)	1.0 (ng mL ⁻¹)	5.0 (ng mL ⁻¹)	20.0 (ng mL ⁻¹)	100.0 (ng mL ⁻¹)
Acephate	n.d. ^a	n.d.	n.d.	-5	-4	5	4
Azoxystrobin	74	53	16	14	1	-1	-3
Bitertanol	n.d.	n.d.	n.d.	n.d.	1	-7	10
Buprofezin	1	4	4	-3	0	-1	-1
Carbaryl	-89	-114	n.d.	-92	-8	-1	0
Chlorpyrifos	38	43	18	2	2	1	3
Chlotianidin	6	-11	9	-2	0	-1	-1
Cyproconazole	3	5	14	1	1	4	3
Difenoconazole	-10	20	-2	8	-1	3	-2
Dimethoate	19	4	0	3	3	2	1
Dimetomorph	53	50	27	25	20	22	17
Diuron	-12	4	-3	2	0	1	-1
Etofenprox	-29	-8	-7	-2	2	3	0
Fenarimol	n.d.	n.d.	n.d.	18	4	2	-1
Fenthion	-2	-2	2	-3	6	-1	2
Fludioxonil	106	430	81	27	0	1	-1
Flufenoxuron	3	9	2	0	4	2	2
Flutriafol	-7	-2	-1	0	1	0	0
Hexaconazole	n.d.	1	-2	0	3	0	0
Imidacloprid	460	191	60	21	7	3	1
Indoxacarb	n.d.	-51	-29	11	4	-3	2
Kresoxim-methyl	n.d.	n.d.	n.d.	-14	-9	-3	-3
Linuron	3	4	-2	4	-1	1	-2
Lufenuron	-25	58	1	-4	-8	4	6
Malathion	22	13	0	5	3	0	0
Methamidophos	0	-7	2	-4	-2	0	-2
Methidathion	60	-17	1	-1	2	0	-1
Methomyl	n.d.	n.d.	n.d.	-19	-7	0	-1

Table 4. Matrix effect calculated for each pesticide by injection of analytical solutions prepared in methanol (n = 7) and in blank matrix extract (n = 7) (cont.)

Pesticide	Matrix effect / %						
	0.1 (ng mL ⁻¹)	0.2 (ng mL ⁻¹)	0.5 (ng mL ⁻¹)	1.0 (ng mL ⁻¹)	5.0 (ng mL ⁻¹)	20.0 (ng mL ⁻¹)	100.0 (ng mL ⁻¹)
Methoxyfenoside	-11	-6	-6	3	2	-3	-1
Omethoate	-3	8	8	-4	0	1	-2
Oxadixyl	n.d.	n.d.	-5	2	1	0	-1
Oxamyl	n.d.	n.d.	-14	4	4	-4	1
Pencycuron	-13	5	4	-3	1	2	-2
Pirimiphos-methyl	1	-6	1	-2	6	0	-2
Prochloraz	n.d.	62	26	2	0	-1	4
Profenofos	-16	9	11	0	-3	2	0
Propiconazole	n.d.	16	1	-1	7	3	-1
Pyraclostrobin	-9	-8	-4	-3	-4	2	-4
Pyriproxyfen	-3	-6	-1	3	2	0	1
Spinosad	5	2	0	-3	-2	-1	-2
Tebuconazole	-1	16	0	5	2	1	-4
Teflubenzuron	-31	19	7	16	8	4	-11
Thiabendazole	5	7	2	2	1	1	-1
Thiacloprid	-4	-1	0	3	2	2	0
Tolyfluanid	n.d.	-23	-12	-7	2	4	-6
Triadimefon	2	6	3	-4	-2	1	-5
Triadimenol	n.d.	n.d.	-3	-2	2	0	0
Trichlorfon	n.d.	n.d.	n.d.	1	-5	-3	-2
Trifloxystrobin	-13	-12	1	-11	0	-2	-1
Triflumizole	n.d.	-35	-9	17	0	2	-2
Zoxamide	9	9	6	5	2	-2	-1

^an.d. = not detected.

Recovery experiments

As can be seen in Figure 3, from the 51 studied pesticides, 45, 46 and 46 had an acceptable recovery percentage in the range from 70 to 120% for spike levels 10, 20 and 50 $\mu\text{g kg}^{-1}$, respectively. One of the pesticides (carbaryl) showed 64% recovery at 10 $\mu\text{g kg}^{-1}$, but at 20 and 50 $\mu\text{g kg}^{-1}$, the recovery values were 85 and 115%, respectively. These values demonstrate the acceptable accuracy of the developed method.

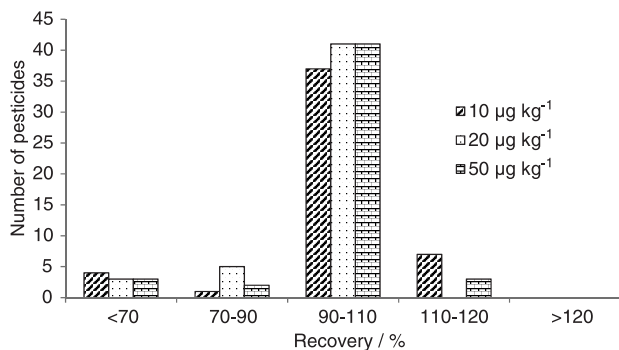


Figure 3. Recoveries (%) for 51 evaluated pesticides spiked on blank tomato at 10, 20 and 50 $\mu\text{g kg}^{-1}$.

Figure 4 shows the RSD values for the analyzed compounds at the studied spike concentrations. As can be seen, only four compounds (carbaryl, kresoxim-methyl, fludioxonil and triadimenol) showed RSD values above 20% at 10 $\mu\text{g kg}^{-1}$. At 20 and 50 $\mu\text{g kg}^{-1}$, only one compound (carbaryl) had an RSD greater than 20%.

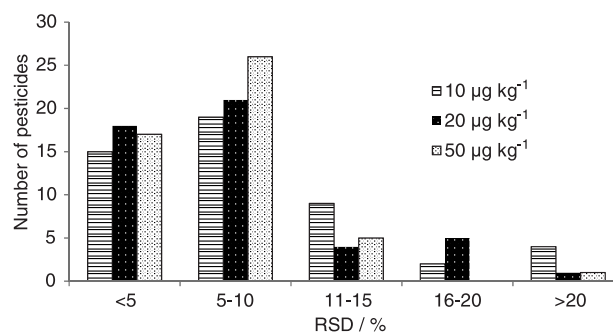


Figure 4. Relative standard deviation (%) results for 51 evaluated pesticides spiked on tomato at 10, 20 and 50 $\mu\text{g kg}^{-1}$.

As for the method LOQ (Figure 5), the validated LOQ_m (showing recovery and RSD values within the acceptable range) was 10 $\mu\text{g kg}^{-1}$ for 42 compounds. For three compounds (kresoxim-methyl, fludioxonil and

triadimenol), the LOQ_m was 20 µg kg⁻¹ and for three others (acephate, methamidophos and omethoate), 50 µg kg⁻¹. For only one compound (carbaryl) the LOQ_m could not be established because the performance criteria were not met, that is, recovery outside 70-120% range and/or RSD > 20% for all three studied concentrations.

Two compounds (bitertanol and triflumizole) were not detected in any of the three spiked concentrations.

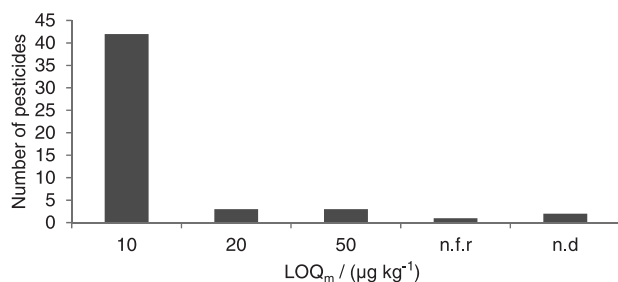


Figure 5. Number of pesticides that showed a LOQ_m of 10, 20 or 50 µg kg⁻¹ (n.f.r. = not fulfilling requirements; n.d. = not detected).

Intermediate precision

Intermediate precision of the developed method was evaluated varying two parameters: different analysts and different days. For all the studied pesticides, the RSD values for intermediate precision were within the acceptable range, except for the spike level of 20 µg kg⁻¹ for carbaryl that showed 78% of RSD.

Figure 6 shows the frequency distribution for the number of compounds, within various RSD ranges, for each spike concentration studied. As can be seen in this figure, only one pesticide (carbaryl) showed an RSD value above 20% at 20 µg kg⁻¹. Two pesticides (bitertanol and triflumizole) were not detected in any of the three spiked concentrations.

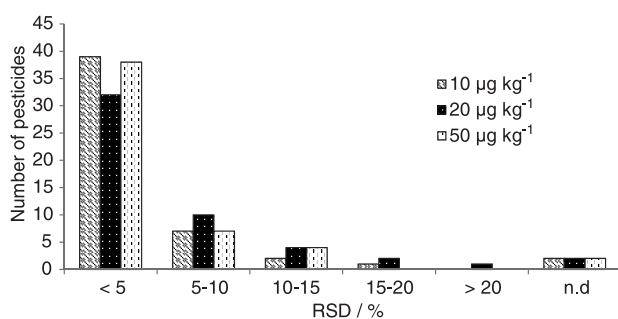


Figure 6. Intermediate precision evaluated via RSD for all studied spiked concentrations (10, 20 or 50 µg kg⁻¹).

Conclusions

Laboratory accreditation provides recognition by official bodies for a laboratory's technical competence in

performing particular services. For the accreditation of testing laboratories, it is necessary to validate methods developed in the laboratory itself to prove its ability to perform certain analyses through validation parameters required by the official accreditation standard for testing laboratories, the ISO/IEC 17025 norm.

For this purpose, a method for determination of 51 pesticides was developed and validated according to the parameters required by the Brazilian accreditation body (INMETRO/CGCRE) and in accordance with ISO/IEC 17025 as a prerequisite for laboratory application to be accredited.

Validation parameters were studied following the official document of Accreditation General Coordination, an agency of INMETRO responsible for laboratories accreditation.

The extraction method used for analysis was the Dutch mini-Luke method based on extraction using acetone and partitioning with petroleum ether and dichloromethane. After the evaporation and reconstitution step in appropriate solvent, the samples were analyzed by ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS).

The validated method showed very good performance for most of the studied analytes in terms of sensitivity, accuracy, precision and linearity except for bitertanol, triflumizole and carbaryl and, for this reason the scope of the method was reduced for 48 pesticides. After all evaluated criteria, the method showed to be appropriate to determine pesticide residues in high water content matrices.

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References

- <http://www.coepbrasil.org.br/portal/Publico/apresentarArquivo.aspx?ID=1efd7f1f-d132-426e-8644-29004872b236> accessed on December 2015.
- Reichert, B.; Pizzutti, I. R.; Costabeber, I. H.; Uclés, A.; Herrera, S.; Fernández-Alba, A. R.; *Talanta* **2015**, *134*, 415.
- Camino-Sánchez, F. J.; Zafra-Gómez, A.; Ruiz-García, J.; Bermúdez-Peinado, R.; Ballesteros, O.; Navalon, A.; Vílchez, J. L.; *J. Food Compos. Anal.* **2011**, *24*, 427.
- Pizzutti, I. R.; Kok, A.; Cardoso, C. D.; Reichert, B.; Kroon, M.; Wind, W.; Righi, L. W.; Silva, R. C.; *J. Chromatogr. A* **2012**, *1251*, 16.
- <http://ilac.org/publications-and-resources/ilac-documents/promotional-brochures/> accessed on December 2015.

6. ISO/IEC 17025: *General requirements for the competence of testing and calibration laboratories*, ISO: Geneva, 2005.
7. <http://ilac.org/publications-and-resources/ilac-documents/promotional-brochures/> accessed on April 2015.
8. http://inmetro.gov.br/credenciamento/sobre_lab.asp accessed on May 2015.
9. Saeger, S. D.; *Determining mycotoxin and mycotoxigenic fungi in food and feed*, 1st ed.; Woodhead Publishing, Cambridge, 2011.
10. SANCO/12571/2013: *Guidance document on analytical quality control and validation procedures for pesticide residue analysis in food and feed*. European Commission: Brussels, 2013.
11. General Inspectorate for Health Protection; *Analytical Methods for Pesticide Residues in Foodstuffs*, 6th ed.; The Netherlands: Ministry of Public Health, Welfare and Sport, 1996.
12. Hiemstra, M.; Kok, A.; *J. Chromatogr. A* **2007**, *1154*, 3.
13. Luke, M. A.; Frober, J. E.; Masumoto, H. T.; *J. AOAC Int.* **1975**, *56*, 1020.
14. DOQ-CGCRE-008: *Orientation of analytical methods validation*, INMETRO: Rio de Janeiro, 2011.
15. Fernández-Alba, A. R.; *Chromatographic-Mass Spectrometric Food Analysis for Trace Determination of Pesticide Residues - Wilson & Wilson's comprehensive analytical chemistry*, 1st ed.; Elsevier: Amsterdam, 2005.
16. Pizzutti, I. R.; Kok, A.; Schoten, J.; Righi, L. W.; Cardoso, C. D.; Rohers, G. N.; Silva, R. C.; *Talanta* **2014**, *129*, 352.
17. Pizzutti, I. R.; Kok, A.; Hiemstra, M.; Wickert, C.; Prestes, O. D.; *J. Chromatogr. A* **2009**, *1216*, 4539.
18. Pizzutti, I. R.; Kok, A.; Zanella, R.; Adaime, M. B.; Hiemstra, M.; Wickert, C.; Prestes, O. D.; *J. Chromatogr. A* **2007**, *1142*, 123.

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