

A Simple and Efficient Method Employing Solid-Liquid Extraction with Low-Temperature Partitioning for the Determination/Monitoring of Pesticide Residues in Strawberries by GC/ECD

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O uso indiscriminado de agrotóxicos em morangos pode torná-los impróprios para consumo. Neste estudo, o método extração sólido-líquido com partição em baixa temperatura (SLE/LTP) acoplado a cromatografia gasosa com detector por captura de elétrons (GC/ECD) foi otimizado. O método proposto foi validado pela análise de amostras fortificadas do branco da matriz. Os limites de detecção (LOD) variaram entre 4 e 16 $\mu\text{g kg}^{-1}$, enquanto que os limites de quantificação (LOQ) variaram entre 13 e 47 $\mu\text{g kg}^{-1}$. Boas recuperações (79-111%) e precisão (< 15%) foram obtidos para todos os compostos na matriz. Este método foi aplicado com sucesso na análise de resíduos de agrotóxicos encontrados em morangos coletados de sete fazendas do Vale do Jequitinhonha (MG, Brasil). Os resultados revelaram a presença de azoxistrobina, clorotalonil, difenoconazol e iprodiona nas amostras de morango.

The indiscriminate use of pesticides in strawberries can often make this crop unfit for consumption. In this study, the method of solid-liquid extraction with low-temperature partitioning (SLE/LTP) coupled with gas chromatography with electron capture detection (GC/ECD) was optimized. The proposed analytical method was validated by the analysis of spiked blank matrix samples. Limits of detection (LOD) varied between 4 and 16 $\mu\text{g kg}^{-1}$, whereas limits of quantification (LOQ) ranged from 13 to 47 $\mu\text{g kg}^{-1}$. Good recoveries (79-111%) and precision values (< 15%) were obtained for all compounds in the target matrix. This method has been successfully applied to the analysis of residues of pesticides found in strawberries collected from seven farms in the High Jequitinhonha Valley (MG, Brazil). The results revealed the presence of azoxystrobin, chlorothalonil, difenoconazole and iprodione in the strawberry samples.

Keywords: multiresidue analysis of pesticides, monitoring, maximum residue levels

Introduction

Strawberry is a widely appreciated fruit worldwide with great acceptability by consumers because of its attractive organoleptic characteristics, such as its typical intense red color, texture, aroma and taste.¹ However, because strawberry plants are sensitive to pests and diseases, pesticide use in this crop is very intense. Additionally,

the use of unauthorized active ingredients in this culture is frequent. For these reasons, the presence of residues above the maximum acceptable limits, aside from residues of unauthorized pesticides, is frequent for this culture in Brazil.²

Obesity is among the diseases that can be caused by chemicals present in crops.³ In large crops, pesticides are sometimes applied by aircrafts and infiltrate the soil to the waterbeds. Moreover, Meyer *et al.*⁴ noted that many of these substances are excreted in breast milk, constituting a

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source of contamination in newborns. An intriguing fact is that many people consume fruits and vegetables to prevent obesity; however, the interference of certain products with hormones can further facilitate the disease. This problem has increased as the selling of pesticides has grown 93% over the last ten years worldwide. In Brazil, this growth was even higher (190%), and between 2001 and 2008,⁵ pesticide sales increased from a little over US\$ 2 billion to US\$ 7 billion, becoming the highest world consumer with 986.5 thousand tons of applied pesticides. In 2010, the national market increased to nearly US\$ 7.3 billion, representing 19% of the global pesticide market, and reached US\$ 8.5 billion in 2011.

The analysis of pesticide residues in foods is not always an easy task because of the complex matrix in which the analytes are present in very small concentrations (ng g^{-1}).⁶ In addition, several extraction stages involving large volumes of organic solvents are generally necessary, making the employed techniques long and extremely painstaking.⁷

For these reasons, solid-liquid extraction with low-temperature partitioning (SLE/LTP) seems to be a viable alternative for analyte extraction of complex matrices. This is related to the greater affinity of the components of interest for the organic phase compared to the aqueous phase and the capacity to avoid components that interfere with the matrix in the final extraction.⁸⁻¹¹ The main advantages of SLE/LTP include low solvent consumption and the absence of cleanup stages for obtaining relatively pure extracts that can be directly analyzed by gas chromatography (GC).

Historically, GC has been the technique selected for multiresidue pesticide analysis in products of vegetable origin, providing results with detection levels ranging from nanograms to micrograms *per kg* (ng kg^{-1} to $\mu\text{g kg}^{-1}$), depending on the detector and extraction technique used.^{12,13} SLE/LTP in combination with GC/electron capture detection (ECD) analysis has been applied to the determination of pesticide residues in soils and foods.^{14,15}

The present study aims to determine the residues of 11 pesticides (azoxystrobin, bifenthrin, cypermethrin, chlorothalonil, chlorpyrifos, difenoconazole, endosulfan, iprodione, λ -cyhalothrin and permethrin) in strawberries from the High Jequitinhonha Valley in Minas Gerais, Brazil using SLE/LTP and GC/ECD.

Experimental

Reagents and solutions

The stock solutions were prepared from standards of azoxystrobin (99.9% m/m), chlorothalonil (99.3% m/m),

difenoconazole (97.0% m/m), endosulfan (73.2% α and 26.6% β m/m), iprodione (99.3% m/m) and permethrin (92.2% m/m) purchased from Sigma-Aldrich (Steinheim, Germany); cypermethrin (92.4% m/m) and chlorpyrifos (99.0% m/m) purchased from Chem Service (West Chester, USA); λ -cyhalothrin (86.5% m/m) purchased from Syngenta (São Paulo, Brazil); and bifenthrin (92.2% m/m) purchased from FMC (Campinas, Brazil) using the like solvent acetonitrile (Vetec/HPLC, Duque de Caxias, Brazil) in concentrations of 1000.0 mg L^{-1} and stored at $-20 \text{ }^\circ\text{C}$.

The working solution containing 10.0 mg L^{-1} azoxystrobin, cypermethrin, chlorothalonil, chlorpyrifos and endosulfan; 20.0 mg L^{-1} bifenthrin, iprodione, λ -cyhalothrin and permethrin and 50.0 mg L^{-1} difenoconazole was prepared by diluting the stock standard solutions in acetonitrile.

Sodium chloride (99.0% m/m) and acetic acid (99.7% v/v) were purchased from Isobar (Duque de Caxias, Brazil).

Gas chromatography conditions

A gas chromatograph (model GC-2014, Shimadzu, Kyoto, Japan) equipped with an auto-injector (AOC-20i), electron capture detection (ECD) system, a DB-5 capillary column (Agilent Technologies, Palo Alto, CA, USA), a stationary phase of 5% phenyl and 95% dimethylsiloxane and a $30 \text{ m} \times 0.25 \text{ mm} \times 0.10 \mu\text{m}$ film thickness was used. The detector temperature was maintained at $300 \text{ }^\circ\text{C}$ and nitrogen (Air Products, São Paulo, Brazil, 99.999% purity) was employed as a carrier gas.

For simultaneous determination of pesticides after extraction, the following column oven temperature program was employed: initial temperature of $150 \text{ }^\circ\text{C}$ (2 min), heating ramp of $40 \text{ }^\circ\text{C min}^{-1}$ to $210 \text{ }^\circ\text{C}$ (2 min), followed by a ramp of $10 \text{ }^\circ\text{C min}^{-1}$ to $250 \text{ }^\circ\text{C}$ (2 min), followed by a ramp of $20 \text{ }^\circ\text{C min}^{-1}$ to $290 \text{ }^\circ\text{C}$ (maintained for 7 min). The injector temperature was maintained at $280 \text{ }^\circ\text{C}$ and the employed carrier gas flow was 1.2 mL min^{-1} . The injected volume was $1.0 \mu\text{L}$ and injections were conducted with a flow split of 1:5. The total analysis time was 20.5 min and the runs were managed by Shimadzu GC-solution software. The pesticides were identified by comparing the retention time of the peak present in the extracts of the samples with the retention time of the standard and were quantified by the matrix-matched method.

SLE/LTP preparation of samples

Samples of pesticide-free strawberries were obtained from an organic cultivation system and ground in a mixer.

Then, 4.0000 g of pulp measured on an analytical balance (Sartorius BP 2215, Göttingen, Germany) with an accuracy of 1×10^{-4} g were added to a clear glass with a capacity of 22.0 mL, which was then spiked with 80 μ L of a working solution containing pesticides.

Spiked samples of strawberry (0.40 μ g g⁻¹ azoxystrobin, cypermethrin, chlorothalonil, chlorpyrifos and endosulfan; 0.80 μ g g⁻¹ bifenthrin, iprodione, λ -cyhalothrin and permethrin and 2.0 μ g g⁻¹ difenoconazole) were allowed to stand for approximately 3 h after the contained mixture was opened. The spiked samples were then subjected to SLE/LTP analysis for the optimization process.

Two full factorial designs were used to optimize the parameters of the SLE/LTP for the analysis of select pesticides in the strawberry samples.

First, a full factorial design, 2², was employed to review the behavior of concurrent factors: solvent extractor and the ratio of sample:extracting solvent on two levels. The analyses were performed in duplicate (Table 1).

Table 1. Factorial designs, 2², performed in duplicate to establish the best conditions for the extraction of pesticides from strawberry samples

Test	Factors (coded levels)		Factors (real values)	
	Solvent extractor	Sample:solvent extractor proportion	Solvent extractor	Sample:solvent extractor proportion
1	-	-	MeCN	1:1
2	+	-	MeCN:EtOAc	1:1
3	-	+	MeCN	1:2
4	+	+	MeCN:EtOAc	1:2
5	-	-	MeCN	1:1
6	+	-	MeCN:EtOAc	1:1
7	-	+	MeCN	1:2
8	+	+	MeCN:EtOAc	1:2

Next, the influence of ionic strength on the extraction efficiency of the analytes in the strawberry samples was assessed by adding an aqueous solution of sodium chloride at a concentration of 2.0% (m/v) to the extractor solvent (acetonitrile), which increased the ionic strength of the medium. The effect of acidification of the medium was also evaluated by acidifying the extractor solvent (acetonitrile) with acetic acid. To assess the behavior of these two factors simultaneously, a second factorial design, 2², was employed. The analyses were performed in triplicate at the central point (Table 2).

The best conditions were assessed for chromatographic responses (areas) obtained in each test at a 95% level of confidence. These data were analyzed using Statistica (version 8) software.

Table 2. Factorial design, 2², performed in triplicate at the center point to establish the best conditions for the extraction of pesticides from strawberry samples

Test	Factors (coded levels)		Factors (real values)	
	Acid addition	Salt addition	Acid addition / %	Salt addition / mL
1	-	-	0	0
2	+	-	1.0	0
3	-	+	0	1.0
4	+	+	1.0	1.0
5C	0	0	0.5	0.5
6C	0	0	0.5	0.5
7C	0	0	0.5	0.5

The sample:solvent extractor mixture was stirred for 15 min in an orbital shaker (Tecnal, model TE-141, Piracicaba, Brazil) at 25 °C with a rotation of 200 rpm, centrifuged (Centrifuge Excelsa II, model 206BL, FANEM, São Paulo, Brazil) for 4 min at a rotation of 617.28 g and allowed to cool in a freezer at approximately -20 °C for 9 h, based on the proposed method from Heleno *et al.*¹⁶ From the supernatant fraction (acetonitrile), 1.5 mL of solution was removed and placed directly into a vial for chromatographic analysis.

Method validation

To evaluate the selectivity of the method, the method was applied to strawberry samples without pesticides and those spiked with pesticides. Subsequently, the chromatograms from these samples were compared. The linearity of the method was evaluated using the analytical curve obtained by analyzing the extracts of the samples spiked with concentrations of pesticides ranging from 0.05 to 0.60 mg kg⁻¹ (n = 7 points) by a linear square regression analysis. For endosulfan, all of the concentrations followed the same isomeric ratios of the standard. The mathematical relationship between the signal (response) and the concentration of the analytes of interest is expressed by the equation of the analytical curve and its determination coefficient (R²). The performance parameters for the method were evaluated, which included selectivity, precision (repeatability), accuracy/recovery, limit of detection (LOD), and limit of quantification (LOQ) of the analytes.

Limits of detection were calculated according to the expression $3.3 \sigma/S$, where σ is the standard deviation of the blank and S is the slope of the calibration curve. Limits of quantification (LOQ) were established by using the expression $10 \sigma/S$.¹⁷

To determine the repeatability, samples were spiked in replicates of six at three different concentrations (0.10, 0.15 and 0.60 mg kg⁻¹). The results are expressed as the coefficient of variation. The accuracy was determined from the recovery assays in which known quantities of the analyte had been added to the sample in replicates of six at three different concentrations (0.10, 0.15 and 0.60 mg kg⁻¹). The results are expressed by the recovery percentage.

Determination of pesticides in strawberries from the High Jequitinhonha Valley in Minas Gerais, Brazil

Strawberry samples were obtained directly from farms in the High Jequitinhonha Valley at three different harvest periods during the same season in 2012: T1, T2 and T3.

The fruits used for the analysis were selected among those collected for sale. The sample collection method followed the Food and Agriculture Organization of the United Nations.¹⁸ The fruits were wrapped in foil and plastic bags, transported in isothermal boxes to the laboratory where they were then ground in a mixer. The matrix extraction of pesticides was performed by SLE/LTP, followed by detection/quantification via GC/EDC.

The fruits originated from seven different farms totaling 21 samples (triplicate), and the results are expressed as triplicate means.

Results and Discussion

Optimization of pesticide extraction

The effects and significance of the variables in the SLE/LTP system were evaluated using Pareto charts (Figure 1).

From the analysis of the effects of these factors on the pesticides, the solvent extractor factor showed a significant effect at the 95% confidence level ($p < 0.05$) over two pesticides (endosulfan- α and iprodione). However, the sample:solvent extractor ratio affected the response for all of the pesticides investigated in this study. Furthermore, reducing the proportion of the extracting solvent to the sample increased the chromatographic response for all of the pesticides. Thus, we chose acetonitrile as the solvent extractor in a 1:1 sample:extractor ratio.

A second full factorial design, 2², was employed to evaluate the effect of adding salt or acid to the extraction of the analytes. The addition of salt to the aqueous phase in the liquid-liquid extraction method using acetonitrile allows for efficient separation of the homogeneous system, improving the extraction of polar compounds.¹⁹ The increase in ionic strength also causes a reduction of the solubility of the

analytes in the matrix, facilitating their extraction by the organic phase. Acidification of the sample may change the ionized form of certain analytes, affecting their solubility and improving the efficiency of the extraction.²⁰ Mařtovská and Lehotay²¹ have shown in their studies that the addition of 0.1% (v/v) acetic acid to acetonitrile increases the stability of pesticides prior to analysis.

The analysis of the effect of these factors on pesticides has shown that both salt and acid addition exhibit a significant effect at the 95% confidence level ($p < 0.05$) on iprodione. Thus, we chose not to add salt or acid in the extraction process.

Taking into consideration the results of the optimization method and the practical aspects of the SLE/LTP technique, a 4.0000 g sample of strawberries in 4.0 mL of acetonitrile was chosen. The mixture was stirred for 15 min on an orbital shaker at 25 °C at a rotation of 200 rpm, centrifuged for 4 min at a rotation of 617.28 g and allowed to cool in a freezer at approximately -20 °C for 9 h. From the supernatant fraction (acetonitrile), 1.5 mL of solution was removed and placed directly into a vial for chromatographic analysis.

The SLE/LTP technique requires low consumption of the sample and solvent extractor because it is not necessary to implement steps of evaporation and solvent exchange, which reduces the risk of contamination and loss of samples.

Method validation

To evaluate the selectivity of the method, the method was applied to strawberry samples without the application of pesticides. Subsequently, these samples were spiked with pesticides and again subjected to the extraction and analysis method. The chromatograms from these samples were then compared. It was observed that there was no interference of the response and the retention time for the analytes of interest. To assess the linearity of the method, seven concentration levels were analyzed, resulting in a determination coefficient (R²) greater than 0.95. The calculated limits of detection (LOD) values for the investigated active ingredients, using GC/EDC, varied from 4 to 16 µg kg⁻¹, and the limits of quantification (LOQ) varied between 13 and 47 µg kg⁻¹ (Table 3).

Among the studied pesticides, iprodione, λ -cyhalothrin, difenoconazole and azoxystrobin are allowed in strawberry culture. The maximum residue limits (MRLs) for these pesticides in fruits, established by the Brazilian Health Surveillance Agency (ANVISA),²² are 2.0, 0.5, 0.5 and 0.3 mg kg⁻¹, respectively, which are above the LOQ values obtained using the technique in this study. Thus,

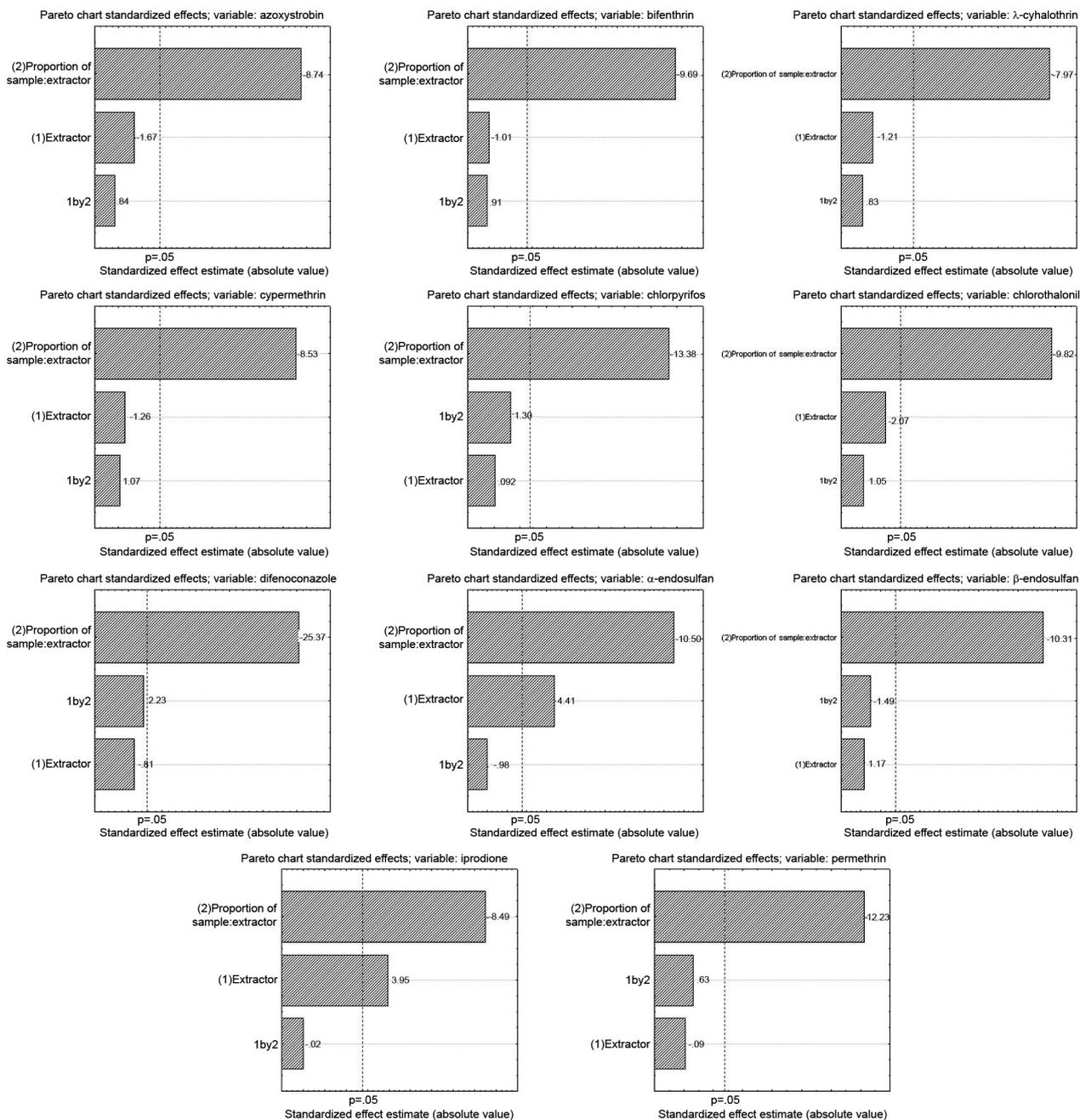


Figure 1. Pareto charts of the full factorial experimental design for the analysis of the two variables: solvent extractor and the proportion of sample:extractor.

the LODs obtained in the study allow for the detection of residues in samples with values below the MRL, which characterizes them as unsatisfactory or satisfactory. For the other evaluated pesticides, simple detection characterizes the samples as unsatisfactory because they are not allowed in this culture.

The precision of this method was determined to range from 1 to 15% for the lowest concentration evaluated (0.10 mg kg^{-1}), 2 to 15% for the middle concentration (0.15 mg kg^{-1}), and 1 to 14% for the highest concentration (0.60 mg kg^{-1}). The recoveries of the pesticides ranged from

79 to 111% when employing the proposed method. These results are summarized in Table 4.

Determination of pesticides in strawberries from the High Jequitinhonha Valley in Minas Gerais, Brazil

The results obtained for the 21 samples from different farms at three collection periods are presented in Table 5. The pesticides chlorpyrifos, α -endosulfan, β -endosulfan, iprodione, λ -cyhalothrin, permethrin and cypermethrin were not detected and were therefore omitted from the table.

Table 3. Linear equation, determination coefficient, limit of detection, limit of quantification and maximum residue limits (MRL) for the pesticides studied

Pesticide	Linear Equation	R ²	LOD / (mg kg ⁻¹)	LOQ / (mg kg ⁻¹)	MLR / (mg kg ⁻¹)
Chlorothalonil	y = 4912206.08x + 578486.50	0.98	0.008	0.025	— ^a
Chlorpyrifos	y = 1956658.39x + 191052.45	0.97	0.004	0.013	— ^a
α-Endosulfan	y = 3049087.25x + 185625.81	0.98	0.004	0.012	— ^a
β-Endosulfan	y = 4545505.16x + 93832.28	0.97	0.006	0.019	— ^a
Iprodione	y = 246403.54x + 9361.98	0.95	0.009	0.027	2.0
Bifenthrin	y = 835271.74x + 525816.95	0.96	0.006	0.018	— ^a
λ-Cyhalothrin	y = 1827084.71x + 112937.64	0.98	0.004	0.011	0.5
Permethrin	y = 316716.28x + 4692.88	0.99	0.016	0.047	— ^a
Cypermethrin	y = 2332747.80x + 185379.68	0.98	0.011	0.033	— ^a
Difenoconazole	y = 792009.30x + 25124.12	0.99	0.015	0.045	0.5
Azoxystrobin	y = 1227698.83x + 88083.61	0.98	0.013	0.039	0.3

^aActive ingredient not allowed in strawberry culture.

Table 4. Precision and accuracy for each analyte obtained from strawberry samples spiked at three different concentration levels^a

Pesticide	Precision / %CV		
	0.10 mg kg ⁻¹	0.15 mg kg ⁻¹	0.60 mg kg ⁻¹
Chlorothalonil	3	11	3
Chlorpyrifos	5	15	2
α-Endosulfan	8	7	5
β-Endosulfan	6	11	5
Iprodione	7	7	14
Bifenthrin	14	9	3
λ-Cyhalothrin	1	11	1
Permethrin	6	2	2
Cypermethrin	15	8	3
Difenoconazole	14	9	3
Azoxystrobin	12	6	1

Pesticide	Accuracy / %RE		
	0.10 mg kg ⁻¹	0.15 mg kg ⁻¹	0.60 mg kg ⁻¹
Chlorothalonil	88	111	98
Chlorpyrifos	95	95	95
α-Endosulfan	93	110	103
β-Endosulfan	84	109	96
Iprodione	92	111	102
Bifenthrin	79	92	103
λ-Cyhalothrin	89	108	99
Permethrin	86	98	101
Cypermethrin	98	107	100
Difenoconazole	102	101	101
Azoxystrobin	91	102	98

^aFor endosulfan, all concentrations followed the same isomeric ratios of the standard. %CV: percent coefficient of variation; %RE: percent relative error.

According to the data in Table 5, residues of the investigated pesticides were not detected in 28.6% of analyzed samples and 33.3% presented residues below

the established MRL. Additionally, 38.1% of the samples were considered unsatisfactory for containing residues of unauthorized products and/or presenting levels above the MRL.

Among the unsatisfactory samples, chlorothalonil was detected in 62.5% of the samples (corresponding to 23.8% of the total analyzed samples), which is an unauthorized active principle for the strawberry crop; 25.0% of the samples (9.5% of the total analyzed samples) presented authorized product concentrations above the MRL and two irregularities were observed in 12.5% of the samples (4.8% total). These results are in agreement with the latest Food Pesticide Residues Analysis Program data,^{22,23} which revealed that the major irregularities are associated with the application of unauthorized active ingredients.

The use of unauthorized pesticides results in two types of irregularities: applying unauthorized pesticides to the crop whose active ingredient is registered in Brazil and is allowed for other crops, or applying pesticides that have been banned in Brazil or have no registration in the country, which are consequently not allowed for use in any crop.²²

Another irregularity is pesticide concentration in fruits above the MRL. This occurrence is disturbing because in some cases, it has been determined that the simple washing of fruits and vegetables is not effective for completely removing the residues of these products. Pesticides can have two action mechanisms in the vegetable: systemic and contact action. Contact action occurs externally to the plant, whereas systemic action occurs when the applied pesticide circulates through the sap in all vegetable tissues and therefore cannot be removed by washing.

The established MRL for azoxystrobin in the strawberry crop is 0.3 mg kg⁻¹. This active principle was detected

Table 5. Pesticide residues in mg kg⁻¹ found in strawberry samples from the High Jequitinhonha Valley in 2012

Active principle		Farm 1	Farm 2	Farm 3	Farm 4	Farm 5	Farm 6	Farm 7
Chlorothalonil ^a	T1	0.91	0.55	0.61	0.10	n.d.	n.d.	n.d.
	T2	0.19	n.d.	n.d.	0.13	n.d.	n.d.	n.d.
	T3	n.d. ^b	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Difenoconazole	T1	n.d.	n.d.	0.10	n.d.	n.d.	0.43	n.d.
	T2	0.12	0.09	0.25	n.d.	n.d.	0.13	0.22
	T3	n.d.	n.d.	< LOQ ^c	n.d.	n.d.	n.d.	n.d.
Azoxystrobin	T1	n.d.	n.d.	< LOQ	0.09	n.d.	n.d.	n.d.
	T2	0.87 ^d	n.d.	< LOQ	n.d.	n.d.	0.34 ^d	0.15
	T3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Iprodione	T1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.13
	T2	n.d.	n.d.	0.68	0.26	n.d.	n.d.	n.d.
	T3	0.18	n.d.	2.16 ^d	0.51	n.d.	n.d.	n.d.

^aActive ingredient not allowed in strawberry culture; ^bn.d. = not detected; ^c< LOQ = below quantification limit; ^dabove legal MRL for strawberry crop.

in 28.6% of the samples, two of which were above the MRL with values of 0.81 and 0.34 mg kg⁻¹. Iprodione was detected in only one sample at 2.16 mg kg⁻¹, slightly above the established MRL of 2.0 mg kg⁻¹.²² Difenoconazole was the most prevalent pesticide found in samples from 5 farms and was determined to be within the limits established by legislation in all of them.

Conclusions

The method developed herein allowed for the simultaneous determination of 11 pesticides in strawberry samples. Satisfactory precision and recovery values were obtained to monitor pesticides at trace levels. Pesticide application in strawberry crops in the High Jequitinhonha Valley region has been determined to be inadequate. Irregularities were found in 38.1% of the strawberry samples produced and sold in that region (pesticide MRL above permitted level and/or unauthorized pesticide for crop). Among the investigated pesticides, residues of azoxystrobin, difenoconazole, iprodione and chlorothalonil were detected and/or quantified, given that the latter is unauthorized for this crop. It can be concluded that awareness measures and training of producers are necessary to prevent the inappropriate use of pesticides to guarantee consumer health.

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