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Solid-Liquid Extraction with Low-Temperature Partitioning as an Alternative to Determinate Pesticide Residues in Teas

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Methods used to determine pesticide residues in teas typically require several laborious steps for sample preparation before analysis. In this study, a simple method based on solid-liquid extraction with low-temperature partitioning (SLE/LTP) and analysis by gas chromatography coupled to mass spectrometry (GC-MS) was optimized and validated for the determination of pesticide residues (pirimiphos-methyl, flutriafol, cyproconazole, and bifenthrin) in dehydrated green tea leaves. After low-temperature partitioning (LTP), the extract in acetonitrile was subjected to a clean-up step using primary secondary amine (PSA) and octadecylsilane (C18) as sorbents for chlorophyll removal. The optimized SLE/LTP-GC-MS method was validated, and it proved to be effective and selective for extracting pesticides from green tea samples, presenting limit of detection (LOD) and limit of quantification (LOQ) of 0.015 and 0.050 mg kg^{-1}, respectively. Recovery ranged between 81-111%, coefficients of variation were less than 16% and coefficients of determination (R^2) were greater than 0.990. The optimized and validated method was applied to green tea and 13 other tea varieties sampled in South and Southeast regions of Brazil. The pesticides under study were detected in some of those samples at values higher than the maximum residue limits (MRLs) allowed by the European Union.

Keywords: SLE/LTP, chromatography coupled with mass spectrometry, method validation, sample preparation, residue analysis, *Camellia sinensis*

Introduction

Originally from Asia, tea has gained widespread popularity and become one of the most consumed beverages worldwide, leading to increased production and exports.[1] Green tea (*Camellia sinensis*) is commonly used due to its benefits, being a powerful ally against diseases because of its wide range of polyphenols and antioxidants, which act as anti-inflammatory agents,[2] reduce the risk of diabetes,[3] and osteoporosis,[4] and prevent cardiovascular diseases.[5]

Because of the positive effects of green tea on health, there has been an increase in its consumption and cultivation. Consequently, many countries have used pesticides to protect crops from certain target organisms and guarantee tea production. Notwithstanding, the excessive use of these pesticides poses a risk to the environment and human health, and their residues can be transferred from the dried tea leaves to the infusions, making tea a significant source of pesticide exposure to humans.[6]

Several countries have established maximum pesticide residue limits (MRLs) for various foods and beverages, including tea. The European Union, for example, has determined the MRLs of hundreds of pesticides and their metabolites in tea products.[7] Thus, it is necessary to devise reliable, robust, and sensitive analytical methods to detect pesticide residues in these matrices.

Developing methods to detect these residues in tea is challenging due to the complexity of the matrix, which contains many polyphenols, pigments, organic acids, caffeine, and other compounds that can interfere with the analytical results.[8] Therefore, sample preparation is essential to remove interferents, making the extract suitable for analysis by instrumental techniques and improving the selectivity of the method.[9]

Various techniques have been employed to determine pesticide residues in tea, such as matrix solid-phase

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dispersion (MSPD),[10] QuEChERS (quick, easy, cheap, effective, rugged, and safe method),[11] dispersive solid-phase extraction (d-SPE),[12] and Soxhlet extraction.[13] Some of these methods have many steps and require large quantities of samples, sorbents, and high-quality organic solvents. Therefore, developing low-cost procedures that generate less environmental contamination is essential.[14]

Low-temperature partitioning (LTP) is a simple technique that has shown excellent results for determining pesticide residues in different matrices, such as foods,[15,16] water,[17] soil,[18] and some biological samples.[19] The method consists of adding water and a miscible water organic solvent to the sample, which is then stored in a freezer at –20 °C for phase separation. The solid or liquid matrix freezes with the water, while the acetonitrile remains liquid and extracts the analytes of interest, forming the upper organic phase. The freezing step also acts as an extract purification step, effectively immobilizing matrix components and particles, with no additional extract cleaning step required for most samples. The organic extract is separated from the frozen phase and analyzed by an appropriate analytical technique. This technique has some advantages, including its feasibility, good selectivity, and reliability, in addition to requiring fewer steps.[20]

Although the freezing step allows simultaneous extraction of the analytes and cleaning of the extract in a single step, for some more complex matrices an additional cleaning step is necessary to remove pigments, dyes, fats, sugars, flavonoids, etc., and reduce the interference of co-extractives in chromatographic analysis.[20] The high levels of chlorophyll in green tea leaves pose a challenge for analysis by gas chromatography (GC) because of the non-volatile characteristic of this compound. When a sample containing chlorophyll is injected into a GC system, the substance accumulates in the liner and may reach the column, forming new active sites. Thus, the determination of pesticide residues in tea leaves requires an additional clean-up step, which is fundamental to diminishing interferences, the matrix effect, and the need for maintenance of the chromatographic system.[21,22] The technique most commonly used in the clean-up step is the d-SPE, employing different types of sorbents, such as primary secondary amine (PSA), octadecyl (C18), and graphitized carbon black (GCB), or a combination of these. The d-SPE technique has the advantage of being simple, fast, and suitable for cleaning extracts from various matrices, including tea.[23,24]

The separation and detection of compounds usually take place in a chromatographic system based on gas chromatography (GC) or liquid chromatography (LC) coupled to different detectors.[25-27] Currently, GC and LC coupled to mass spectrometry (GC-MS and LC-MS) are the most commonly used methods for pesticide-residue analysis in tea, making it possible to obtain low limits of detection (LOD) and quantification (LOQ).[28-30]

Many methods have been reported in the literature for monitoring pesticide residues in green tea. Huang *et al*.,[31] for example, applied the QuEChERS method to green tea and detected some type of pesticide in 67% of the samples. Wu *et al*.[26] used a modified version of the QuEChERS method to check pesticides in green tea and found that 65% of the samples were contaminated. In those studies, the pesticide concentrations were above the MRL allowed by the European Union, and some samples contained substances unauthorized or even prohibited for this crop. These findings highlight the need to monitor pesticide residues in this matrix.

On that account, this study aimed to optimize and validate a new method for determining four different pesticides (pirimiphos-methyl, flutriafol, cyproconazole, and bifenthrin) in dehydrated green tea leaves through solid-liquid extraction with low-temperature partitioning (SLE/LTP) followed by clean-up via d-SPE and analysis by GC-MS. The validated method was then applied to green tea and different types of tea obtained in the South and Southeast regions of Brazil. To the best of our knowledge, this is the first time that SLE/LTP was applied to determine pesticides in tea, and this technique is simpler than others described in the literature.

Experimental

Reagents and solutions

The experiments used analytical standards of the pesticides pirimiphos-methyl (99.5%), flutriafol (97.0%), cyproconazole (99.8%), and bifenthrin (92.2%), which were obtained from Sigma-Aldrich (Seelze, Germany). Stock solutions from each pesticide standard were prepared in high-performance liquid chromatography (HPLC)-grade acetonitrile (99.9%) (Sigma-Aldrich, Burlington, USA) at a concentration of 1000 mg L^{-1}. The other working solutions were prepared from the stock solutions by dilution in acetonitrile. All the solutions were kept in a freezer at approximately –20 °C. The experiments also employed the sorbents PSA (Agilent Technologies, São Paulo, Brazil), C18 (Supelco, São Paulo, Brazil), GBC (Supelco, São Paulo, Brazil), chitosan (Sigma-Aldrich, São Paulo, Brazil), silica (Merck, São Paulo, Brazil), florisil (Sigma-Aldrich, São Paulo, Brazil), and sodium sulfate (Na_2SO_4) (Vetec, São Paulo, Brazil).

Samples

Dehydrated pesticide-free green tea samples were purchased from the local market in Viçosa (Minas Gerais, Brazil). They were used to optimize and validate the SLE/LTP technique associated with d-SPE. The absence of pesticides was confirmed by GC-MS. Other tea variety samples (green, black, white, chamomile, mint, lemon balm, fennel, horsetail, "*erva-mate*", "*boldo*", dandelion, "*espinheira santa*", stonebreaker, and lemongrass) were purchased in some cities in the South and Southeast regions of Brazil. They were also subjected to the proposed method.

Chromatographic settings

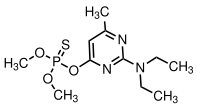
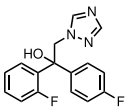
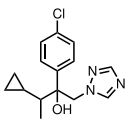
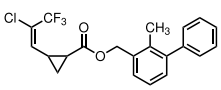
The chromatographic analyses were performed with a gas chromatograph equipped with a quadrupole mass-spectrometry detector (MS) (Shimadzu, model GCMS-QP2010, Kyoto, Japan) and an auto-injector (Shimadzu, model AOC-20i, Kyoto, Japan). The analytical conditions optimized for the chromatographic separation of the analytes included a capillary column (30 m × 0.25 mm × 0.25 μm, SH-Rtx-5MS) with a stationary phase composed of 5% phenyl and 95% dimethylpolysiloxane. Helium 99.999% purity (White Martins, Rio de Janeiro, Brazil) was used as the carrier gas at a flow rate of 1.17 mL min^{-1}. The column temperature started at 160 °C for 1 min, then it ramped at a rate of 20 °C min^{-1} up to 290 °C, at which it was kept for 2.5 min. The injector temperature was maintained at 300 °C, and 1 μL of the sample was injected into the chromatograph using the splitless injection mode. The total analysis time was 10.0 min.

The MS operated with an electron impact of 70 eV. A cut-off time of 5.0 min and interface temperatures at the detector, the ionization sources, and mass analyzer (single quadrupole) of 300, 230, and 150 °C, respectively, were set to avoid instrumental damage.

Initially, an investigative analysis was performed to obtain the retention times (t_R) and fragmentation profile of the pesticides. The mass spectra were obtained in the *m/z* range from 50 to 500 in SCAN mode. After that, high-intensity fragment ions guided the confirmation and quantification of the pesticides and their elution times in the chromatogram using the selected ion monitoring (SIM) mode.

The physicochemical properties of the pesticides pirimiphos-methyl, flutriafol, cyproconazole, and bifenthrin and their respective monitored ions, retention time, chemical group/class, toxicologic class, and maximum residue limits (MRLs) according to the European Union are shown in Table 1.

Table 1. Physicochemical properties of the pesticides pirimiphos-methyl, flutriafol, cyproconazole, and bifenthrin and their respective monitored ions, retention time, chemical group/class, toxicologic class, and maximum residue limits (MRLs) according to the European Union

Compound	Monitored ions	Retention time / min	Chemical group / class	Toxicologic class	Molecular weight / (g mol^{-1})	Log K_{ow}[a]	Solubility[b] / (mg L^{-1})	Boiling point[c] / °C	MRL[d] / (mg kg^{-1})
Pirimiphos-methyl 	290, 276, 305	5.96	organophosphate / insecticide, acaricide	class III	305.33	4.19	11	386.5 ± 52.0	0.05
Flutriafol 	123, 164, 219	6.98	triazole / fungicide	class III	301.29	2.30	95	506.0 ± 60.0	0.05
Cyproconazole 	222, 139, 125	7.39	triazole / fungicide	class III	291.78	3.08	93	476.9 ± 55.0	0.05
Bifenthrin 	181, 166, 165	8.38	pyrethroid / insecticide, acaricide	class III	422.88	6.59	0.001	453.2 ± 45.0	30.0

[a]Octanol-water partition coefficient at pH 7 and 20 °C; [b]in water at 20 °C; [c]at 760 mmHg; [d]maximum residue limit (MRL) according to the European Union[7] and International Union of Pure and Applied Chemistry.[32]

Optimization of the SLE/LTP technique for determining pesticide residues by GC-MS

Sample fortification

Samples of dehydrated, pesticide-free green tea leaves (blank) were used to optimize and validate the method SLE/LTP-GC-MS.

The green tea samples were stored in sealed plastic bags at room temperature to prevent contamination. Following the experimental protocol performed in triplicate, 1.0 g of green tea leaves were weighed with an analytical balance (AUY 220, Shimadzu) and fortified with 0.1 mL of the working solution containing the four pesticides at 32 mg L^{-1}. Next, the mixture was stirred in a vortex for 10 s and allowed to stand for 2 h to enhance the interaction between the pesticides and the matrix and facilitate the evaporation of the organic solvent. Lastly, the samples were subjected to extraction and analysis by GC-MS.

Optimization of SLE/LTP parameters

The optimization of the SLE/LTP-GC-MS method was divided into three stages. The first stage, the removal of chlorophyll by d-SPE from green tea leaf extracts obtained by SLE/LTP was evaluated. Chlorophyll, the pigment responsible for the green coloration in leaves, possesses properties that can compromise the chromatographic system; thus, a clean-up step is required to decrease its content in the extracts. A study to reduce the chlorophyll content in the extract by d-SPE was conducted in three substages: sorbent study, sorbent proportion, and optimization of the d-SPE parameters. All assays were performed in triplicate.

A univariate study tested silica, chitosan, florisil, PSA, C18, GBC, and the mixtures PSA/C18 (1:1, m/m), PSA/C18/GBC (1:1:1, m/m/m), PSA/GBC (1:1, m/m), and C18/GBC (1:1, m/m) for removing chlorophyll from the extracts by d-SPE. An aliquot of 1.5 mL of each extract obtained via SLE/LTP was transferred to 15.0 mL Teflon vials containing 125 mg of each sorbent or sorbent mixture. The system was vortexed (3 min) and then centrifuged (4 min). After the clean-up, the supernatant was collected and diluted in acetonitrile at a 1:4 v/v ratio (extract:solvent). The extracts were analyzed using a spectrophotometer (USB2000+, Ocean Optics, São Paulo, Brazil) operating at 660 nm.

The sorbent that provided the highest percentage of chlorophyll removal was adopted in the subsequent trials, that is, use of PSA/C18 sorbents. The ratios PSA/C18 (1:1, m/m), PSA/C18 (1:3, m/m) and PSA/C18 (3:1, m/m) were subjected to univariate analysis. The cleaning procedure described above was used and the extracts were analyzed in a spectrophotometer operating at 660 nm.

In parallel, a study evaluated potential analyte retention by the PSA/C18 sorbent mixture (1:1, m/m). This study was performed using a SLE/LTP extract and a standard solution in acetonitrile, both fortified with pesticides at a concentration of 0.80 mg L^{-1}. These samples (extract and standard solution) were analyzed in triplicate by GC-MS before and after d-SPE clean-up. The results obtained for the standard solution and SLE/LTP extract without the clean-up step were used as a reference in this study.

A 2^3 experimental design with a central point (n = 3) (Table 2) was applied to optimize the values of the sorbent mixture mass, stirring time, and centrifugation time for the d-SPE technique. The extracts were analyzed in a spectrophotometer (660 nm), and the resulting absorbances were used as analytical responses in each experiment. The resulting data were analyzed using the OriginPro® software (v8, 2007).[33]

The second stage of optimization of the SLE/LTP-GC-MS method used a factorial design 2^3 with a center point to evaluate the variables volume of water (3, 4, or 5 mL), the volume of extracting solvent (4, 5, or 6 mL), and ionic strength (by adding Na_2SO_4 at 0.00, 0.01 or 0.02 mol L^{-1}) was used.

In the third stage, the variables vortex stirring time (30, 45, or 60 s), centrifugation time at 560 g (3, 6, or 9 min), and freezing time at −20 °C (4, 5, or 6 h) were evaluated. A complete factorial design 2^3 with a center point (n = 3) was applied for this purpose. In both designs, the chromatographic areas were used as the analytical response.

SLE/LTP-GC-MS procedure optimized

In glass bottles containing 1.0 g of crushed, sieved green tea leaf samples, 3.0 mL of ultra-purified water in the Milli-Q system were added along with 4.0 mL of acetonitrile and an ionic strength of 0.020 mol L^{-1} using Na_2SO_4. The mixture was then vortexed (Phoenix, AP 56, Araraquara, Brazil) for 30 s, centrifuged (Quimis®, ISO 8001, São Paulo, Brazil) for 3 min at 560 g, and stored in a freezer (Consul, Minas Gerais, Brazil) at −20 °C for 4 h. After separating the phases by freezing the leaves along with the aqueous phase, 1.5 mL of the supernatant obtained was collected and transferred to a vial containing a 125 mg mixture of PSA/C18 sorbents (1:1). This vial was then vortexed for 1 min and centrifuged for 4 min at 560 g. The supernatant was collected and analyzed by GC-MS.

Method validation

Once optimized for determining pesticides in dehydrated green tea leaves, the SLE/LTP-GC-MS

Table 2. Factorial design 2^3 with a center point (n = 3) and evaluated factors: sorbent mass, stirring time, and centrifugation time of the d-SPE clean-up of green tea leaf extracts obtained by SLE/LTP

Variable	Level		
	(−1)	(0)	(+1)
Total sorbent mass / mg	75	100	125
Stirring time / min	1	2	3
Centrifugation time / min	2	3	4
Assay	Coded factor		
	Sorbent mass	Stirring time	Centrifugation time
1	−	−	−
2	+	−	−
3	−	+	−
4	+	+	−
5	−	−	+
6	+	−	+
7	−	+	+
8	+	+	+
9	0	0	0
10	0	0	0
11	0	0	0

method was validated according to the criteria established by the Brazilian Health Regulatory Agency (ANVISA) in Resolution RDC166/2017.[34] The following figures of merit were assessed: selectivity, linearity, LOD, LOQ, accuracy (recovery tests), precision (repeatability and intermediate precision), and matrix effect. All tests were carried out in triplicate.

Application of SLE/LTP-GC-MS to commercial tea samples

The validated SLE/LTP-GC-MS method was applied to determine pesticide residues in dehydrated green tea leaf samples. Subsequently, the applicability of the SLE/LTP-GC-MS method was investigated across 13 different tea varieties. A total of 51 tea samples obtained in the South and Southeast regions of Brazil were evaluated. These included eight samples of green tea (*Camellia sinensis*), three of black tea (*Camellia sinensis*), three of white tea (*Camellia sinensis*), seven of lemon balm (*Melissa officinalis*), five of fennel (*Pimpinella anisum*), seven of chamomile (*Matricaria recutita*), six of horsetail (*Equisetum giganteum*), two of "erva-mate" (*Ilex paraguariensis*), four of "*boldo*" (*Peumus boldus*), two of mint (*Mentha* sp.), and one sample each of dandelion (*Taraxacum officinale*), "*espinheira santa*" (*Maytenus ilicifolia*), stonebreaker (*Phyllanthus niruri*), and lemongrass (*Cymbopogon citratus*).

Results and Discussion

Application of d-SPE for chlorophyll removal from extracts obtained from green tea leaves via SLE/LTP

After submitting the samples to LTP, it was noticed that the extracts contained high chlorophyll content. Therefore, adding a clean-up step to the method was necessary to prevent damage to the chromatographic system and to obtain improved results.

Several sorbents and sorbent mixtures were tested (in triplicate) to evaluate chlorophyll removal from the extracts resulting from the SLE/LTP. The absorbance of the green tea leaf extract obtained without the clean-up step (corresponding to 100% chlorophyll) was compared with that of each extract subjected to clean-up. Figure 1 contains the percentage of chlorophyll removal from the green tea leaf extracts using the different sorbents.

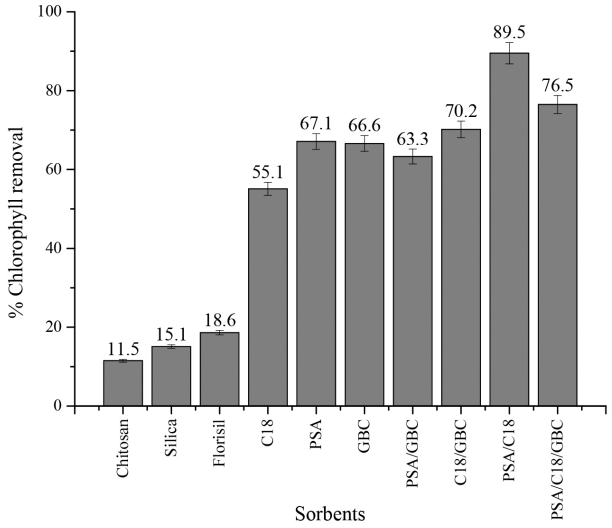


Figure 1. Percentage of chlorophyll removal by d-SPE from green tea leaf extracts obtained by SLE/LTP using different sorbents.

Among the sorbents and mixtures of sorbents tested, PSA/C18 had the most promising results, achieving 89.5% chlorophyll removal. Sorbents such as PSA and C18 are commonly used in the clean-up steps of tea matrices and oily matrices, such as olives and almonds.[35-37]

When tested individually, the sorbents PSA and C18 exhibited lower efficiency compared to their combination. This suggests that their mixture allows for a complementary action, resulting in a more effective removal of chlorophyll from the medium. C18 is recognized for its effectiveness in extracting nonpolar and moderately polar substances. On the other hand, PSA functions as an anion exchanger and can interact with other compounds through hydrogen bonds or dipole-dipole interactions. Furthermore, due

to the presence of primary and secondary amino groups in its structure, PSA can also serve as a chelating agent, making it more suitable for extracting polar compounds. Chlorophyll, on the other hand, contains both polar and nonpolar groups in its structure, enabling it to interact with both sorbents.[38] This provides a rationale for the enhanced extraction efficiency observed when PSA and C18 were combined for chlorophyll removal via d-SPE.

After this stage, a second study was conducted to define the best PSA/C18 ratio. The results are exhibited in Figure 2.

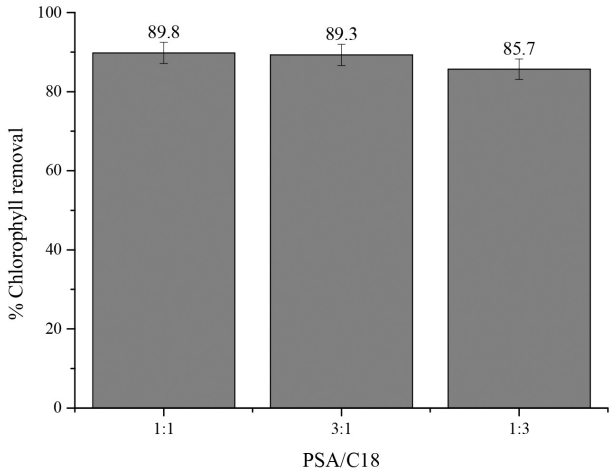


Figure 2. Different PSA/C18 ratios used in the clean-up step to remove chlorophyll from green tea leaf extracts obtained by SLE/LTP.

The results for the three evaluated proportions showed minimal difference in chlorophyll removal. Increasing the amount of PSA conferred greater polarity to the sorbent mixture but did not significantly impact the percentage of chlorophyll removal. Conversely, increasing the proportion of C18 lowered the polarity of the sorbent mixture and reduced its removal efficiency, indicating less interaction between the chlorophyll molecule and the sorbents in the medium. Consequently, PSA/C18 at a ratio of 1:1 (m/m) was chosen for the following stages of this study.

The subsequent experiments investigated whether this sorbent mixture could remove the pesticides of interest. To achieve this purpose, the standard solution and the extract fortified with the analytes were submitted for clean-up and analysis by GC-MS. The areas of the analytes in the standard solution and the extract without clean-up were compared with the corresponding areas of the processes with the clean-up step (Figure 3).

The recovery percentage of the analytes from the standard solution after d-SPE clean-up ranged between 94-98%, while the recovery percentage from the extract submitted to SLE/LTP varied between 107-119%. These

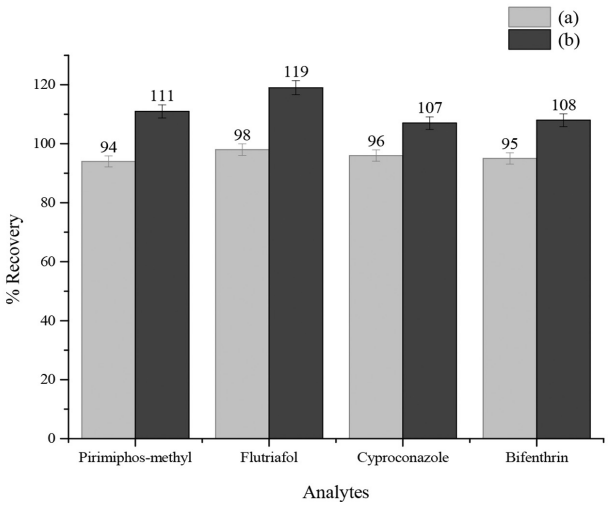


Figure 3. Recovery percentage of each analyte from (a) the standard submitted to d-SPE with a mixture of C18/PSA; (b) green tea extracts obtained by SLE/LTP and submitted to d-SPE with a mixture of C18/PSA.

values exceeding 100% are likely attributed to some matrix interference. The results indicate good recovery, as values between 70-120% are deemed acceptable.[39]

For the optimization of the d-SPE technique as a clean-up step, some parameters for chlorophyll removal were evaluated using an experimental design 2^3 with a center point. The parameters sorbent mass (at a 1:1 ratio; 75, 100, 125 mg), stirring time (1, 2, 3 min), and centrifugation time (2, 3, 4 min) were optimized. The significance of each factor was assessed via analysis of variance (ANOVA) using *p*-value significance levels. The absorbance of each assay was used to evaluate the results and generate a Pareto chart of the effects and interactions. This chart, displayed in Figure 4, enables a comparison of the significance of the effects individually and their interactions, as indicated by the length of the bars.

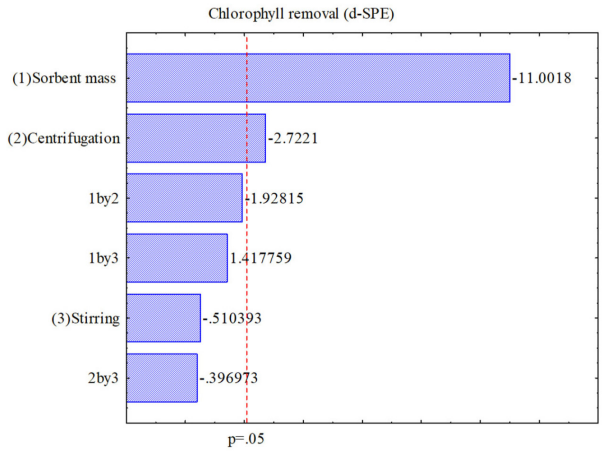


Figure 4. Pareto chart of the effects of the variables (1) sorbent mass, (2) centrifugation time, and (3) stirring time on the process of chlorophyll removal via d-SPE from green tea extract obtained by SLE/LTP.

According to the Pareto chart, stirring (factor 3) was non-significant, whereas sorbent mass (factor 1) and centrifugation time (factor 2) were negatively significant. This suggests that increasing the sorbent mass and prolonging the centrifugation time resulted in a decreased absorbance response. Therefore, the highest efficiency in chlorophyll removal from the green tea extracts obtained by SLE/LTP was achieved when using the largest mass of sorbent (125 mg) at a ratio of 1:1 (62.5 mg PSA, 62.5 mg C18), along with the longest centrifugation time (4 min) and the shortest stirring time (1 min) during the clean-up step.

Figure 5 exhibits the color difference between the extracts from green tea samples obtained by SLE/LTP, either with or without clean-up, using a PSA/C18 mixture.

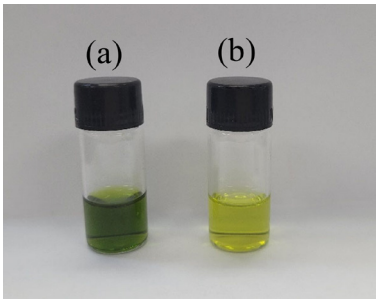


Figure 5. Green tea extracts obtained by SLE/LTP technique (a) without clean-up and (b) with clean-up using a PSA/C18 mixture.

Optimization of the SLE/LTP technique for determination of pesticide residues by GC-MS

The SLE/LTP-GC-MS method was optimized for ionic strength, water volume, and acetonitrile volume parameters, using a 2^3 factorial design with a central point (n = 3). The significance of each factor was assessed via analysis of variance (ANOVA) with a significance level of 95% (*p*-value). The area of each pesticide in the respective tests was used to generate Pareto charts (Figure S1, SI section). Table 3 contains the values of the effects of each variable and their interaction on the optimization of the SLE/LTP-GC-MS method applied to green tea leaves.

The results indicated that the volume of acetonitrile (factor 3) negatively influenced the response of all analytes, whereas ionic strength (factor 1) had a significant positive effect on all of them. These results suggest that the extraction efficiency of the four analytes of interest is enhanced by using a small volume of acetonitrile and high ionic strength.

Different effects were observed concerning the volume of water: it was negative for bifenthrin and positive for the other pesticides. This observed behavior may be attributed to the pesticides' octanol-water partition coefficients (log K_{ow}), which range from 2.30 for flutriafol to 6.59 for bifenthrin (Table 1).

In the SLE/LTP technique, the solid sample is placed in contact with water and a water-miscible extracting solvent (single phase). The ratio of these solvents influences the transfer of analytes from the solid sample (tea leaves) to the single phase, before the freezing step. A higher water percentage promotes the transfer of more polar compounds, and conversely, which could account for the study's observed outcomes.

Due to these different effects observed further investigation was conducted to understand the impact of choosing a small or large water volume on the average signal of the experimental design (constant term). It was found that using a large volume of water decreased the signal of the bifenthrin by 51% on the average signal of the experimental design (constant term). On the other hand, a smaller volume of water decreased the other pesticide signal by up to 29%. Therefore, employing a smaller volume of water proved more advantageous, as it led to less loss of the chromatographic response of the bifenthrin.

In summary, to achieve the best recovery of analytes from green tea leaf samples by SLE/LTP-GC-MS, thereby improving the sensitivity of the method, this study used 3.00 mL of ultra-purified water, 4.00 mL of acetonitrile, and an ionic strength of 0.02 mol L^{-1}.

Subsequently, the parameters of time (stirring, centrifugation, and freezing) were optimized. The experimentally determined area of each analyte was used as the response to generate Pareto charts (Figure S2, SI section). The values found for each parameter are shown in Table 4.

Table 3. Values of optimizing effects from 2^3 design for (1) ionic strength, (2) water, and (3) ACN, and their interactions (1by2, 1by3 and 2by3) of the SLE/LTP-GC-MS method applied to green tea leaves

Analyte	(1) Ionic strength	(2) Water	(3) ACN	1by2	1by3	2by3
Pirimiphos-methyl	4.97	2.43	–20.7	NS	–2.68	2.78
Flutriafol	6.78	8.21	–34.4	NS	–3.27	NS
Cyproconazole	6.51	9.08	–35.5	NS	–3.95	NS
Bifenthrin	2.53	–8.95	–4.26	NS	NS	6.94

ACN: acetonitrile; NS: non-significant.

Table 4. Values of optimizing effects from 2^3 design for (1) stirring, (2) centrifugation, and (3) freezing, and their interactions (1by2, 1by3 and 2by3) of the SLE/LTP-GC-MS method applied to green tea leaves

Analyte	Stirring (1)	Centrifugation (2)	Freezing (3)	1by2	1by3	2by3
Pirimiphos-methyl	–2.34	NS	NS	NS	NS	NS
Flutriafol	NS	NS	NS	NS	NS	NS
Cyproconazole	–2.26	NS	NS	NS	NS	NS
Bifenthrin	NS	NS	NS	NS	NS	NS

NS: non-significant.

The results demonstrated that the stirring time (factor 1) had a negative significant influence on the response of the pesticides pirimiphos-methyl and cyproconazole, but it was not statistically significant for the others (flutriafol and bifenthrin). The factors centrifugation time (factor 2) and freezing time (factor 3) were not statistically significant for the analytes tested.

That being so, the shortest stirring time was chosen, as it was negatively significant for two pesticides and non-significant for the others. As for the centrifugation and freezing times, since they did not show significance for any of the analytes, the shortest durations were adopted in the following experiments. Therefore, the parameters were set at 30 s of stirring, 3 min of centrifugation, and 4 h of freezing.

Validation of the analytical method

Selectivity

The selectivity of the method was checked by comparing the chromatograms of pesticide-free green tea extracts (blank) with those of green tea extracts fortified with the pesticides of interest at 0.8 mg L^{-1} (Figure 6). The samples were subjected to the optimized SLE/LTP procedure, and the extracts were analyzed by GC-MS.

A comparison between the chromatograms confirmed that the green tea extract did not exhibit any interfering compounds at the same retention times as the analytes.

LOD and LOQ

The LOD was determined using a signal-to-noise ratio of 3:1, representing the lowest concentration of fortified matrix extract that provided a signal three times greater than the background noise (blank) at the same retention time of each analyte. The LOQ was calculated with a signal-to-noise ratio of 10:1, representing the lowest concentration that generated a signal ten times greater than the noise at the same retention time of each analyte. The LOD value of the method for all analytes in the study was 0.015 mg kg^{-1}, while the LOQ was 0.050 mg kg^{-1}. The MRL value allowed by the European Union for the pesticides analyzed is equal to the LOQ obtained in this study.[7]

Linearity

The optimized SLE/LTP-GC-MS method was then applied to green tea leaf samples fortified with the pesticides at different concentrations (n = 6). The levels tested ranged from 0.05 to 1 mg kg^{-1} of pirimiphos-methyl, flutriafol, cyproconazole, and bifenthrin. Analytical curves were plotted for each compound, and linearity was assessed

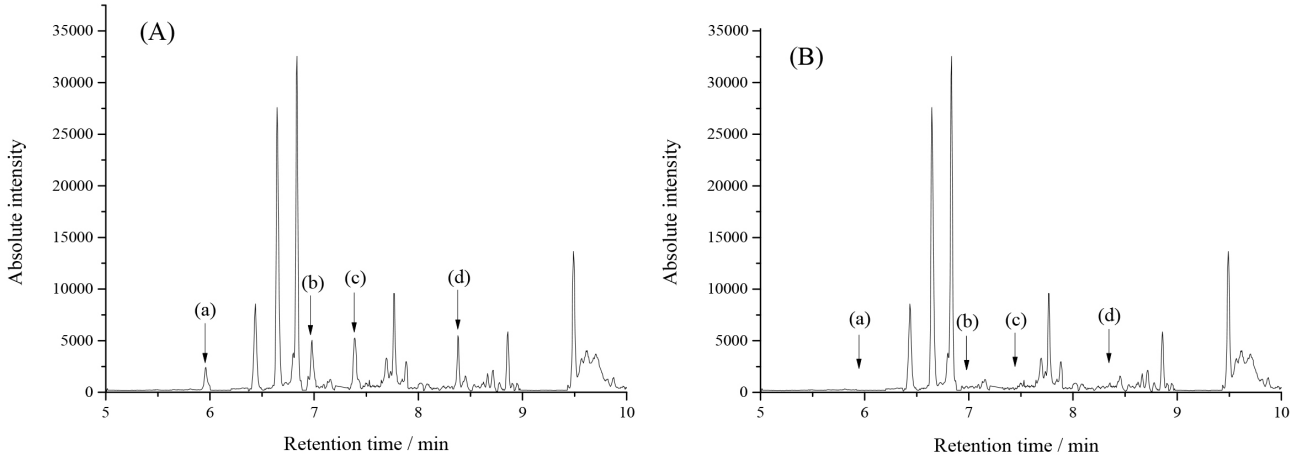


Figure 6. Chromatograms obtained by GC-MS of green tea leaf extracts (A) containing the pesticides (a) pirimiphos-methyl (t_R = 5.96 min), (b) flutriafol (t_R = 6.98 min), (c) cyproconazole (t_R = 7.39 min), and (d) bifenthrin (t_R = 8.38 min); (B) free from these analytes.

through the least squares method and the residue graphs (Figure S3, SI section).

The coefficients of determination (R^2) obtained for the four compounds (Table 5) ranged from 0.991 to 0.997, indicating that the method had good linearity in the concentration range studied being within the range established by the ANVISA parameters.[33] These results were confirmed by residual graph plots obtained for each pesticide (Figure S3, SI section). They revealed a random distribution of the data, confirming the linearity of the method for all four pesticides within the specified range.

Accuracy and intra- and inter-day precisions

The accuracy of the SLE/LTP-GC-MS method was determined through recovery assays carried out in triplicate at three different concentrations (0.05, 0.40, and 1.00 mg kg^{-1}). The recovery percentage ranged from 81-107% (Table 5).

Intra- and inter-day precisions were assessed in triplicate at these same concentrations, considering the coefficients of variation (CVs). The intra-day precision (repeatability) showed a CV between 0.26-10.3% (Table 5). The intermediate precision (inter-day), evaluated on different days, yielded results ranging from 2.42-14.6% (Table 5), indicating good inter-day precision of the data. In complex samples, CVs of up to 20% are generally acceptable, meaning that this study demonstrated good repeatability of the data.

Matrix effect

The matrix effect was evaluated by comparing the analytical curves of the standard solutions prepared in acetonitrile with those of the organic extracts of green tea leaves obtained immediately after the SLE/LTP. The matrix effect percentage (ME) was calculated according to equation 1.[40]

$$\mathrm{ME}\,(\%) = \left[\frac{\mathrm{aE} - \mathrm{aS}}{\mathrm{aS}}\right] \times 100 \quad (1)$$

where aE is the slope of the analytical curve of each pesticide in matrix extract, and aS is the slope of the analytical curve of each pesticide in pure solvent.

A positive matrix effect (pirimiphos-methyl 96.4%, bifenthrin 88.4%, flutriafol 45.7%, cyproconazole 28.0%) was observed for all pesticides, indicating an increase in the chromatographic response. This increase can be attributed to the presence of co-extractives in the tea sample extract.

Interactions between the analytes and the active sites of the injector liner (inserter) are the primary cause for the matrix effect on chromatographic analyses. A positive matrix effect occurs when the analytes dissolved in a pure solvent are more retained in the active sites of the inserter than the analytes in the matrix extract, causing them to be transferred to the column in smaller quantities. In contrast, analytes in the matrix extract compete with co-extractives for the inserter active sites, allowing a greater quantity of pesticide to be introduced into the column. When the detector response attributed to the analyte is compared with the response of standard solutions of the same analyte, there is an overestimation of the results, generating a positive effect or an increase in the chromatographic response.[41] In order to minimize the matrix effect on pesticide analyses, pesticide

Table 5. Parameters of method validation for pesticides in green tea leaf samples: regression equation, coefficient of determination (R^2), linear range, limits of detection and quantification (LOD and LOQ), fortification level (FL), recovery percentage and standard deviation (R ± SD), intra- and inter-day coefficient of variation (CV)

Compound	Linear regression / R^2	Linear range	LOD / (mg kg^{-1})	LOQ / (mg kg^{-1})	FL / (mg kg^{-1})	R ± SD / %	Intra-day CV (n = 3) / %	Inter-day CV (n = 9) / %
Pirimiphos-methyl	$y = 19323x + 381.56$ 0.994	0.05-1	0.015	0.05	0.05	81.0 ± 0.003	5.76	9.23
					0.4	103± 0.011	2.60	3.94
					1	99.4 ± 0.019	1.91	2.42
Flutriafol	$y = 59714x - 364.52$ 0.997	0.05-1	0.015	0.05	0.05	83.2 ± 0.001	1.51	15.8
					0.4	110 ± 0.001	0.26	2.95
					1	106 ± 0.036	3.55	2.99
Cyproconazole	$y = 32258x + 1660.6$ 0.996	0.05-1	0.015	0.05	0.05	82.6 ± 0.039	9.75	14.6
					0.4	111 ± 0.418	4.20	8.69
					1	105 ± 0.993	10.3	6.53
Bifenthrin	$y = 88875x + 1102$ 0.991	0.05-1	0.015	0.05	0.05	87.8 ± 0.002	4.04	11.1
					0.4	107 ± 0.029	6.78	8.31
					1	104 ± 0.033	3.21	2.71

quantification was carried out using analytical curves constructed with the matrix extract.

The optimized and validated SLE/LTP-GC-MS method demonstrated simplicity and efficiency in analyzing pesticides in green tea leaves. It involves fewer steps and consumes less organic solvent compared to other methods.[8,26,31]

According to the literature, the QuEChERS method and its modified versions are the most commonly used for determining pesticide residues in teas. Ly *et al.*[8] proposed a method involving QuEChERS followed by an analysis using ultra efficiency liquid chromatography coupled to mass spectrometry (UPLC-MS/MS) and GC-MS/MS for detecting volatile and non-volatile pesticides in green tea. The authors achieved a LOQ below 0.010 mg kg^{-1}, a CV inferior to 15.9%, and recovery rates between 70%-120%. Another study conducted by Huang *et al.*[31] to determine pesticides in green tea, followed by LC-MS/MS analysis, was performed, achieved a LOQ of 0.05 mg kg^{-1}, a CV of less than 18%, and a recovery rate between 71-114%.

In those cases, the use of a more sensitive detector is evident, which explains the slightly lower LOQ found in the first study. However, in the second case, the LOQ was close to the result obtained in this study. All other parameters are similar; thus, the LTP method can be employed as an alternative for determining pesticide residues in teas due to its simplicity and the fewer steps required for sample preparation. Additionally, it has the advantage of applying to solid[18] and liquid[17] samples.

Application of SLE/LTP-GC-MS to commercial tea samples

The validated SLE/LTP-GC-MS method was applied to determine the pesticides pirimiphos-methyl, flutriafol, cyproconazole, and bifenthrin in green tea leaves and in different tea samples sourced from the Southern and Southeastern regions of Brazil. All samples were analyzed in triplicate.

The pesticide pirimiphos-methyl was found above the MRL established by the European Union (0.05 mg kg^{-1}) in five of the tea samples (one black tea, one white tea, and three lemon balm tea) at concentrations ranging from 0.06-0.6 mg kg^{-1}. Flutriafol was verified in one sample of horsetail tea, albeit below the LOQ and, consequently, below the MRL. Cyproconazole was present in two lemon balms, one fennel, one horsetail, and one mint tea sample at concentrations ranging from 0.11-0.85 mg kg^{-1}, all exceeding the MRL. Bifenthrin was found in two green teas, one horsetail, one "*erva-mate*", and one lemongrass tea sample at concentrations varying from below the LOQ to 0.20 mg kg^{-1}. Figure 7 shows the chromatogram of a commercial sample of green tea containing residues of the pesticide bifenthrin (0.20 mg kg^{-1}).

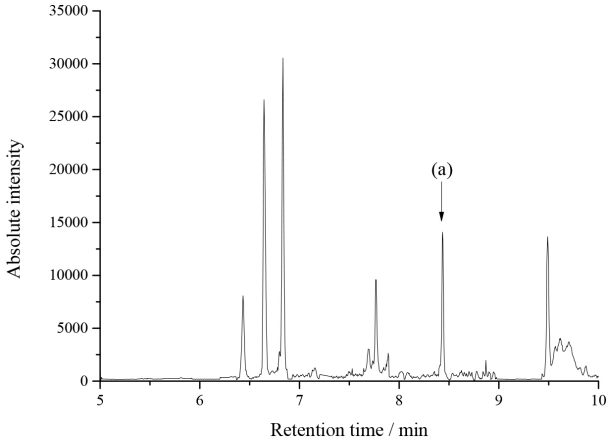


Figure 7. Chromatogram of a commercial sample of green tea containing the pesticide (a) bifenthrin.

These findings highlight the importance of pesticide residue analysis in teas, as evidenced by the positive detections in some samples. Notably, several samples even surpassed the limits set by the European Union. These results also show the applicability of the SLE/LTP-GC-MS method to determine pesticide residues not only in green tea but in other types of teas. However, to obtain the appropriate experimental conditions for each type of tea, the method must be validated or even subjected to new optimization and validation steps.

Conclusions

The optimized and validated method (SLE/LTP) followed by a clean-up step via d-SPE proved efficient for determining the pesticides pirimiphos-methyl, flutriafol, cyproconazole, and bifenthrin in green tea leaf samples by GC-MS. The method delivered high recovery percentages (81-111%), good repeatability using low volumes of sample and solvent, and a limit of quantification of 0.050 mg kg^{-1}. Furthermore, the proposed method is simpler than others listed in the literature, and it can be applied to different tea varieties.

The pesticides in the study were found in many of these commercial tea samples. Some of them contained pesticide residues above the MRL allowed by the European Union. This result highlights the need for monitoring these pesticides in tea samples by regulatory agencies.

Supplementary Information

Supplementary information is available free of charge at http://jbcs.sbq.org.br as PDF file.

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Author Contributions

All the authors contributed to the work presented in this paper. Aline Maria Teixeira was responsible for the conceptualization, data curation, investigation, methodology, validation, and writing; Maria Eliana L. R. de Queiroz contributed to the conceptualization, data curation, formal analysis, fundraising, investigation, methodology, project administration, resources, supervision, validation, visualization, and writing; André F. de Oliveira contributed to the conceptualization, data curation, formal analysis, investigation, methodology, and validation; Alessandra A. Z. Rodrigues contributed to the data curation, investigation, and writing; Fábio Junior M. Novaes contributed to the data curation, investigation, and writing; Jéssika F. de Freitas contributed to the formal analysis, methodology, and writing; Liany D. L. Miranda contributed to the formal analysis, methodology, and writing.

References

1. Hassan, M. M.; Li, H., Ahmad, W.; Zareef, M.; Wang, J.; Xie, S.; Wang, P.; Ouyang, Q.; Wang, S.; Chen, Q.; *LWT - Food Sci. Technol.* **2019**, *105*, 290. [Crossref]
2. Souza, N. C.; Nascimento, O. N. E.; de Oliveira, I. B.; Oliveira, H. M. L.; Santos, E. G. P.; Mata, M. E. R. M. C.; Gelain, D. P.; Moreira, J. C. F.; Dalmolin, R. J. S.; Pasquali, M. A. B.; *Biomed. Pharmacother.* **2020**, *128*, 110277. [Crossref]
3. de Amorim, L. M. N.; Vaz, S. R.; Cesário, G.; Coelho, A. S. G.; Botelho, P. B.; *J. Funct. Foods* **2018**, *40*, 589. [Crossref]
4. Shen, C. L.; Yeh, J. K.; Cao, J. J.; Chyu, M. C.; Wang, J. S.; *Pharmacol. Res*. **2011**, *64*, 155. [Crossref]
5. Chieng, D.; Kistler, P. M.; *Trends Cardiovasc. Med.* **2022**, *32*, 399. [Crossref]
6. Zhu, J.; Yu, J.; Zhang, B.; Li, C.; Wang, J.; Ji, J.; Liu, D.; Gao, R.; Li, J.; Ma, Y.; *Food Chem.* **2023**, *412*, 135546. [Crossref]
7. European Commission (EU); *Pesticides Database*, https://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/start/screen/mrls, accessed in August 2024.
8. Ly, T. K.; Ho, T. D.; Behra, P.; Nhu-Trang, T. T.; *Food Chem*. **2020**, *326*, 126928. [Crossref]
9. Smith, R. M.; *J. Chromatogr. A* **2003**, *1000*, 3. [Crossref]
10. Cao, Y.; Tang, H.; Chen, D.; Li, L.; *J. Chromatogr.* **2015**, *998*, 72. [Crossref]
11. Kardani, F.; Jelyani, A. Z.; Dahanzadeh M.; Rashedinia, M.; *Appl. Food Res*. **2022**, *2*, 100197. [Crossref]
12. Cheng, X.; Yan, H.; Wang, X.; Sun, N.; Qiao, X.; *Food Chem.* **2014**, *162*, 104. [Crossref]
13. Fiedler, H.; Cheung, C. K.; Wong, M. H.; *Chemosphere* **2002**, *46*, 1429. [Crossref]
14. Kristenson, E. M.; Ramos, L.; Brinkman, U. A. T.; *TrAC, Trends Anal. Chem.* **2006**, *25*, 96. [Crossref]
15. Heleno, F. F.; Rodrigues, A. A.; Queiroz, M. E. L. R.; Neves, A. A.; Oliveira, A. F.; Libardi, V. M.; *Microchem. J.* **2019**, *148*, 79. [Crossref]
16. de Ávila, M. B. R.; Faroni, L. R. A.; Heleno, F. F.; de Queiroz, M. E. L. R.; Almeida, R. I.; *J. Braz. Chem. Soc*. **2020**, *31*, 1991. [Crossref]
17. Goulart, S. M.; Alves, R. D.; Neves, A. A.; de Queiroz, J. H.; de Assis, T. C.; de Queiroz, M. E. L.; *Anal. Chim. Acta* **2010**, *671*, 41. [Crossref]
18. Ramírez, A. C. R.; Teixeira, M. F. F.; Neves, A. A.; Queiroz, M. E. L. R.; da Silva, A. A.; Furtado, I. F.; de Oliveira, A. F.; *J. Exp. Agric. Int.* **2018**, *21*, 1. [Crossref]
19. Silva, T. L. R.; de Queiroz, M. E. L. R.; de Oliveira, A. F.; Rodrigues, A. A. Z.; Neves, A. A.; Vieira, P. A. F.; de Queiroz, J. H.; Barbosa, V. O. P.; *J. Environ. Sci.* **2022**, *57*, 165. [Crossref]
20. Goulart, A. C.; Rodrigues, A. A. Z.; Heleno, F. F.; de Faria, A. M.; Goulart, S. M.; Queiroz, M. E. L. R.; *Anal. Chim. Acta* **2024**, 342795. [Crossref]
21. de Pinho, G. P.; Silvério, F. O.; Neves, A. A.; de Queiroz, M. E. L. R.; *Quim. Nova* **2012**, *35*, 738. [Crossref]
22. Mol, H. G. J.; Rooseboom, A.; Dam, R.V.; Roding, M.; Arondeus, K.; Sunarto, S.; *Anal. Bioanal. Chem*. **2007**, *389*, 1715. [Crossref]
23. Chen, L.; Zhang, Y.; Zhou, Y.; Li, G. H.; Feng, X. S.; *J. Chromatogr. A* **2021**, *1644*, 462068. [Crossref]
24. Liu, X.; Guan, W.; Hao, X.; Wu, X.; Ma, Y.; Pan, C.; *Chromatographia* **2014**, *77*, 31. [Crossref]
25. Wang, Q.; Wang, M.; Jia, M.; She, Y.; Wang, J.; Zheng, L.; El-Aty, A. M. A.; *J. Chromatogr. A* **2023**, *1705*, 464209. [Crossref]
26. Wu, Y.; An, Q.; Li, D.; Kang, L.; Zhou, C.; Zhang, J.; Pan, C.; *Food Chem*. **2022**, *375*, 131819. [Crossref]
27. Ramezani, S.; Mahdavi, V.; Gordan, H.; Rezadoost, H.; Conti, G. O.; Khaneghah, A. M.; *Environ. Res.* **2022**, *208*, 112730. [Crossref]
28. Soltani, S.; Sereshti, H.; *Food Chem.* **2022**, *380*, 132181. [Crossref]
29. Song, L.; Zeng, W.; Li, A.; Pan, C.; Pan, L.; *Food Control*. **2022**, *131*, 108436. [Crossref]
30. Sajid, M.; Alhooshani, K.; *Microchem. J.* **2020**, *152*, 104464. [Crossref]

31. Huang, Y.; Shi, T.; Luo, X.; Xiong, H.; Min, F.; Chen, Y.; Nie, S.; Xie, M.; *Food Chem*. **2019**, *275*, 255. [Crossref]
32. International Union of Pure and Applied Chemistry (IUPAC); *Pesticide Properties Database*, https://sitem.herts.ac.uk/aeru/iupac/atoz.htm, accessed in August 2024.
33. *OriginPro*, version 8; OriginLab Corporation, Northampton, MA, USA, 2007.
34. Agência Nacional de Vigilância Sanitária (ANVISA); Resolução da Diretoria Colegiada (RDC) No. 166, de 24 de julho de 2017, *Dispõe sobre a Validação de Métodos Analítico se dá Outras Providências*, Diário Oficial da União (DOU), Brasília, No. 141, de 25/07/2017, p. 87. [Link] accessed in August 2024
35. Balkan, T.; Kara, K.; Yılmaz, Ö.; Kızılarslan, M., Özbek, Ö. F.; *Food Chem. Adv.* **2023**, *3*, 100442. [Crossref]
36. López-Blanco, R.; Nortes-Méndez, R.; Robles-Molina, J.; Moreno-González, D.; Gilbert-López, B.; García-Reyes, J. F.; Molina-Díaz, A.; *J. Chromatogr. A* **2016**, *1456*, 89. [Crossref]
37. Steiniger, D.; Lu, G.; Butler, J.; Phillips, E.; Fintschenko, Y.; *J. AOAC Int.* **2010**, *93*, 1169. [Crossref]
38. Liu, X.; Xu, J.; Dong, F.; Li, Y.; Song, W.; Zheng, Y.; *Anal. Bioanal. Chem.* **2011**, *401*, 1051. [Crossref]
39. Ribani, M.; Bottoli, C. B. G.; Collins, C. H.; Jardim, I. C. S. F.; Melo, L. F. C.; *Quim. Nova* **2004**, *27*, 771. [Crossref]
40. Kwon, H.; Lehotay, S. J.; Geis-Asteggiante, I.; *J. Chromatogr. A* **2012**, *1270*, 235. [Crossref]
41. Albero, B.; Sánchez-Brunete, C.; Tadeo, J. L.; *Talanta* **2005**, *66*, 917. [Crossref]

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