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ARTICLE

Multiresidue analysis of pesticide in peanuts using modified QuEChERS sample preparation and Liquid Chromatography-Mass Spectrometry detection

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A multiclass method has been optimized and validated for the simultaneous determination of 113 pesticides residues belonging to several classes in peanuts. It has been based on QuEChERS methodology (quick, easy, cheap, effective, rugged and safe) and ultra high performance liquid chromatography coupled to triple quadrupole tandem mass spectrometry (UHPLC–MS/MS). Several extraction solutions were tested and the composition that showed the best results consisted of a mixture of ethyl acetate and acetonitrile. A cleanup step using dispersed phase C18 (octadecyl) and PSA (Primary and Secondary Amine) was necessary due to the high amount of oil present in the matrix. The method was validated and the parameters of validation were satisfactory. The accuracy was assessed by calculating the recovery of spiked blank samples in four concentration levels (0.010; 0.025; 0.050 and 0.100 mg kg^{-1}). The results showed satisfactory recoveries (between 70 - 120 %), except for oxamyl and tricyclazol at 0.010 mg kg^{-1} level that did not show acceptable parameters for the recovery assays. Repeatability and intermediate precision aggraded showed coefficients of variation < 20%, except for buprofenzin, etione and picolinafem at 0.100 mg kg^{-1} level. Limits of detection and quantification of the method were 0.005 and 0.010 mg kg^{-1}, respectively, except for oxamyl and tricyclazol.

Introduction

The cultivation of peanuts can be affected by many diseases and thus different types of pesticides are applied to crops for their protection. These compounds are widely applied in a variety of different ways during the production of foods to growth control of weeds and fungi or to prevent crop damage by insects, mites, rodents, and other pests. Pesticides are also frequently used in crops postharvest to prolong storage life and improve quality [1]. However, the harmful effects of extensive use of these contaminants on human health have been the subject of several studies. Unlike other toxic chemicals, the pesticides are designed to kill, repel, or otherwise, harm living organisms and they are one of the few toxic substances that are intentionally applied to the environment [2]. These organic toxins enter animal and human bodies directly or indirectly through the food chain or drinking water.

Regulatory guidelines set maximum residue levels (MRLs) in drinking water and food to help protecting people against contamination and potential negative health effects. Then, the MRLs list for a wide variety of commodities and pesticides is updated from time to time, it is part of the EU Plant Protection Products Directive (2005/396/EEC)[3] and (2009/1107/EEC)[4] which is the update of the former directive (91/414/EEC)[5]. In Brazil, the Ministry of Agriculture considers the ensuring of food safety as one of its duties to further the development of programs that promote improvement of the quality of food for domestic consumption and also for export. So, the Ministry of Agriculture published the Normative Instruction nº 42 of 31 December 2008[6] establishing the National Control Plan for Residues and Contaminants (PNCRC) for products of vegetal origin. Thus, to meet the current Brazilian legislation's requirements, it is necessary the development of specific and sensitive methods for the determination of pesticides in food.

Pesticides, depending upon their water solubility can either remain in the soil where they are broken down by action of microorganisms, or washed off, eventually into surface waters and groundwater[7]. In view of potential toxic and persistent nature of some pesticides, there is the pressing need for their control and monitoring in the environment and food [8,9]. The analysis of pesticides in food is considered to be a difficult task, due to the high complexity and diversity of the matrices, the matrix components and low concentrations in which these compounds are usually present [9]. Moreover, peanuts are recognized as a difficult matrix to deal due to their high oil content. In the food control analysis, isolation of pesticides from matrices containing relatively high fat content, such as peanut oil, requires complicated sample treatment procedures. The preparation of these samples for determination of pesticides by chromatographic methods requires the complete removal of the high molecular weight fat before sample introduction into chromatographic column[10]. Commonly, liquid-liquid extraction, gel permeation chromatography (GPC), or low-temperature fat precipitation have been used as a post extraction cleanup procedure for fatty matrices [9,11]. Solid-phase extraction (SPE), microwave assisted extraction (MAE), matrix solid-phase dispersion (MSPD) and the new SPE technique based on carbon nanotubes have also been applied to oily matrices for extraction and cleanup[12]. However, these methods often require large solvent volumes; use a lot of glassware, and take much time and labor, which reduce the laboratory efficiency and sample throughput. In this context, an alternative proposal of sample preparation for analysis of pesticides known as QuEChERS (an acronym for Quick, Easy, Cheap, Effective, Rugged and Safe) was introduced by Anastassiades and coworkers[13]. Nowadays this methodology has been used around the

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world in many studies for residue analysis of pesticide and/or other compounds in different matrix samples including dried samples[8,14].

Liquid chromatography–mass spectrometry (LC–MS) allows the rapid and efficient determination of many pesticides compounds, as pesticides, for example[15]. There are different mass analyzers that can enhance tandem mass spectrometry (MS/MS) capabilities, such as quadrupole ion-trap (QLIT), triple quadrupole (QqQ) and quadrupole time-of-flight (QTOF) – each one has different features. The main advantage of QqQ instruments is their very good quantitative capabilities and their great sensitivity in the selected reaction monitoring (SRM) mode, in addition to the capability of simultaneously selecting multiple transitions[16].

In this context, this work aims to develop, optimize and validate a method for analysis of 113 pesticide residues in peanut (belonging to 46 pesticide classes: aryloxyalkanoic acid/ester, aryloxyphenoxypropionate, pyridinecarboxylic acid/ester, acylalanine, anilinopyrimidine, avermectin, benzamide, benzimidazole, benzofuran, benzothiazinone, carbamate, carbamate oxime, carboxamide, cyanoacetamide oxime, cyanoimidazole, chloroacetamide, diacylhydrazine, dicarboximide, dinitroaniline, sulfite ester, strobilurin, phenylamide, phenylpyrazole, phenylpyridazin, phosphorothiolate, hidroxianilide, imidazole, isoxazole, methylcarbamate, morpholine, neonicotinoid, organophosphate, oxadiazine, piperazine, pyrazole, pyrethroid, pyridazinone, pyridine, pyridinecarboxamide, pyrimidine, sulphamide, sulfonylurea, thiocarbamate, triazinilsulfonilureia, triazole and urea) aiming to meet the demand of PNCRC monitoring program in Brazil. Peanut samples were submitted to a modified QuEChERS extraction and sequentially submitted to a selective and sensitive LC-MS/MS analysis. Validation of the method was made based on the European Union SANCO/12571/2013 guidelines[17] and Manual of Analytical Quality Assurance from MAPA[18].

Experimental

Materials and reagents

All reagents were of analytical grade. HPLC-grade acetonitrile and glacial acetic acid were supplied by Merck (Darmstad, Germany). Methanol was obtained from Baker (Xalostoc, México). Anhydrous magnesium sulfate (purity of 97%) was purchased from Sigma-Aldrich while anhydrous sodium acetate PA and ammonium acetate (purity of 98%) were purchased from Vetec (Rio de Janeiro, RJ), respectively. Formic acid was purchased from Tedia (Ohio, USA). Ultrapure water was generated by a Millipore Milli-Q system (Milford, MA, USA). All the standards were of high purity grade (>98.0%) and were purchased from Riedel-de Haën (Selze, Germany) or Sigma-Aldrich (Saint Louis, USA). Individual stock solutions were prepared at 1000 mg L^{-1} in acetonitrile or methanol and stored at -20 ± 2 ^{o}C in a freezer. The working solutions were prepared as appropriate dilutions of the stock solutions.

Instrumentation

Chromatographic conditions

Chromatographic analyses were performed using an UHPLC system equipped with a binary pump (Shimadzu LC20ADXR), an auto sampler (Shimadzu SIL20ACXR) and a column oven (Shimadzu CTO20AC). The separations were achieved using a Shim-pack XR-ODSII column (2.0 x 100 mm, 2.2 µm particle size). Chromatographic separation was carried out with a mobile phase consisting of ammonium acetate (10 mmol.L^{-1}) acidified with 0.01% formic acid (phase A) and methanol (phase B) at a flow rate of 0.5 mL min^{-1}. The gradient elution program was as follows: A (50%)–B (50%) (1 min), A (20%)–B (80%) (6 min), A (10%)–B (90%) (4 min), A (50%)–B (50%) (0.5 min), and A (50%)–B (50%) (1.5 min). The total chromatographic run time was 13 minutes. Injection volume was 5 µL and the column temperature was set at 60 ^{o}C.

Mass spectrometric conditions

Mass spectrometry analysis was carried out using a 5500 Triple Quadrupole mass spectrometer (AB Sciex, Ontario, Canada). The instrument was operated using electrospray ionization (ESI) in positive and negative ion modes. Instrument settings, data acquisitions and processing were controlled by the software Analyst (Version 1.5.1, AB Sciex). Source parameters were optimized as follows: ion spray voltage, 5.5 kV for ESI (+) and 4.5 kV for ESI (-); curtain gas, 20 psi; collision gas, 8 psi; nebulizer gas and auxiliary gas, 30 and 30 psi, respectively; ion source temperature, 500 °C. Optimal declustering potential (DP), collision energy potentials (CE) and collision exit potentials (CXP) are shown in the Table 1 (Supplemental Information).

Sample preparation

The blank peanut samples were obtained from local supermarkets (Belo Horizonte, Brazil). The samples were previously ground and stored at 4°C until analysis. Blank samples were fortified with target compounds during the optimization and validation of the developed procedure. Pesticides were extracted from peanut using an extraction procedure based on QuEChERS methodology. The procedure was as follows: 2.5 g of the sample was weighted in a polypropylene tube followed by the addition of 7.5 mL of water and 10.0 mL of a mixture of acetonitrile:ethyl acetate:acetic acid (49:50:1, v/v/v) solution. Then, the mixture was stirred by vortex for 1 min. Afterwards 4.0 g of anhydrous magnesium sulfate and 1.0 g of sodium acetate was added and the tubes were shaken for 1 min again. After centrifugation at 4000 rpm for 9 min, the system was frozen at -20 °C for 2 hours. After that, 1 mL of the organic layer was subjected to a cleanup with 150 mg of anhydrous magnesium sulfate, 75 mg of dispersive C18 and 25 mg of PSA (primary and secondary amine). The system was vortexed for about 1 min. and ultra-centrifuged at 9000 rpm for 8 min. Finally, 500 µL of the extract was injected into UHPLC–MS/MS system.

Method validation

The method validation was performed according to Document Nº SANCO/12571/2013 guidelines [17]. Analytical parameters evaluated were mean recovery (as a measure of trueness), repeatability and intermediate precision (as a measure of precision), limit of detection, limit of quantification and measurement uncertainty.

Selectivity and calibration curves

The selectivity of the method was evaluated by injecting extracted blank samples. The absence of signal above a signal-to-noise ratio of 3 at the retention times of the target compounds showed that the method is free of interferences. Matrix-matched calibration (MMC) was used in order to minimize the matrix effect because matrix constituents may increase or decrease the analytical signal. For the preparation of analytical MMC curves, blank peanut extracts were spiked with proper amounts of standard solutions at the final concentrations of 0.0050; 0.0075; 0.0100; 0.0250; 0.0500; 0.0750; 0.1000 mg kg^{-1} (where this sequence was randomly injected (n = 6)). All solutions were prepared independently. For simultaneous quantification and identification purposes, two SRM transitions for each analyte (Table 1, Supplemental Information) were used in order to avoid false negatives at traced pesticide levels. The integration of all chromatographic peaks was checked by using the Analyst software (Version 1.5.1, AB Sciex). Statistical treatment of generated data was done by means of an in-house developed spreadsheet. The best type of fit regression curve was decided for

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each compound by applying the homoscedasticity test. The Ordinary Least Squares method (OLS) was used for the homocedastic data, while Weighted Least Squares Method (WLS) was used for heteroscedastic data. The fit quality and significance of the regression model employed were evaluated using the Lack of Fit test. The significance level used in all tests was 95%.

Trueness and precision

The trueness was determined from the recovery assay results of samples spiked with all the analytes at four distinct levels: 0.010; 0.025; 0.050 and 0.100 mg kg^{-1} (n = 6 replicates per level) on three different days by two analysts and quantifying the measurement uncertainty (MU). Repeatability, expressed as relative standard deviation (RSD), was evaluated through the data from replicates samples (n = 6) analyzed at same day for each level. The intermediate precision, expressed as relative standard deviation (RSD), was evaluated through the replicates data (n = 18) in the three different days for each level.

Limit of detection, limit of quantification and measurement uncertainty

The limit of detection (LOD) was experimentally determined by spiked blank peanut extracts with all the analytes. The LOD was defined as the lowest concentration of analyte that could be differentiated of the matrix signal with a signal-to-noise ratio (*S/N*) greater than 6. The LOQ was based on the trueness and precision data, obtained via the recovery determinations and was defined as the lowest validated spike level meeting the requirements of a recovery within the range 70 – 120 % and a RSD ≤ 20%. Measurement uncertainty (MU) was accessed according to ISO/TS 21748:2004 [19] and EURACHEM guide [20].

Results

Optimization of instrumental conditions

UHPLC coupled to MS/MS is the most suitable technique for the simultaneous determination of multi-class pesticides, allowing the reliable analysis of this type of compounds at low levels in complex matrices. Accordingly, the first step in the development of the method consisted in the optimizing instrumentals conditions. In this sense, for MS/MS detection, ESI in positive ion mode was used, and two more intense transitions (quantification and confirmation transitions) per compound were monitored. The MS/MS parameters for each compound are shown in Table 1 (Supplemental Information). Then, the chromatographic conditions were studied in order to provide overall optimum peak shape and resolution. Thus, the mobile phase composition was investigated to maximize the method sensitivity and resolution. The mobile phase consisted of a gradient of ammonium acetate (10 mmol L^{-1}) acidified with formic acid (0:01% v/v) and methanol as described above. The total ion chromatograms (TIC) are shown in Figure 1.

Extraction Optimization

Parameters such as sample size and extraction solution volume were based on previous studies involving other matrices[21]. Firstly, 20 of the 113 analytes validated were randomly selected to optimize the extraction process through the evaluation of the recovery results, because the analysis of all compounds would require much time. For the other compounds in this stage, the analytes selected were: allethrin, barban, bifenthrin, carbofuran, deltamethrin, spiromesifen, hexaconazole, iprodione, isoproturon, malathion, metazachlor, methiocarb, oxadixyl, ethyl parathion, penconazole, quinalphos, sulfotep, tebuconazole, temephos and thiacloprid.

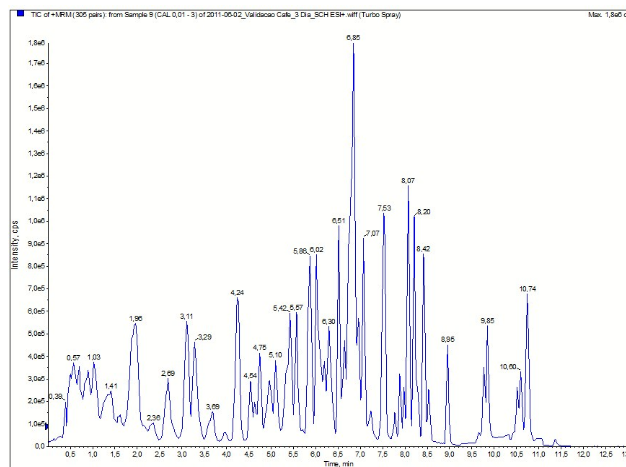

Figure 1. Total ion chromatogram (TIC) obtained by LC–MS/MS (ESI positive mode) for blank peanut extracts spiked with all the analytes at 10.0 μg L^{-1}.

The blank peanuts were fortified in two concentration levels: 0.01 and 0.1 mg kg^{-1}, and they were subjected to extraction tests. The first test consisted in using the acetonitrile solution containing 0.1% acetic acid as extraction solution. The remaining steps of the extraction process were identical to that described above. As can be seen in Figure 2, 45 and 33% of the results were unsatisfactory using this solution at the levels 0.01 and 0.1 mg kg^{-1}, respectively.

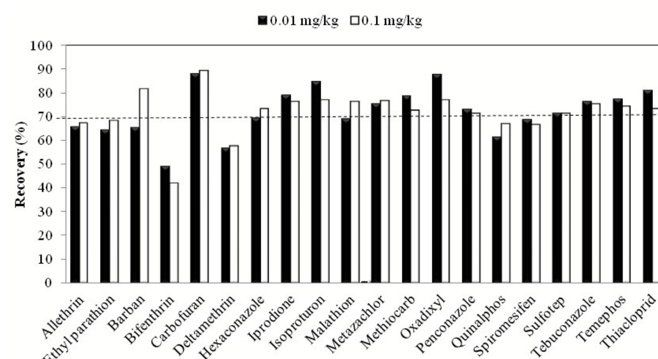

Figure 2. Recovery range of analytes allethrin, barban, bifenthrin, carbofuran, deltamethrin, spiromesifen, hexaconazole, iprodione, isoproturon, malathion, metazachlor, methiocarb, oxadixyl, ethyl parathion, penconazole, quinalphos, sulfotep, tebuconazole, temephos and thiacloprid using acetonitrile solution containing 0.1% acetic acid as extraction solution.

The analytes allethrin, barban, bifenthrin, deltamethrin, spiromesifen, ethyl parathion and quinalphos, that showed recovery less than 70%, are less polar compounds. Then, to solve this problem, new extractions solutions were tested. The second test consisted in the use of three different extraction solutions: (1) ethyl acetate: acetonitrile: acetic acid (10:89:1 v/v/v), (2) ethyl acetate: acetonitrile: acetic acid (30:69:1, v/v/v), (3) ethyl acetate: acetonitrile: acetic acid (50:49:1 v/v/v). As can be seen in Figure 3, the extraction solutions showed 82.5, 87.5 and 92.5% of the satisfactory results, respectively. Thus, the third solution was selected for method validation.

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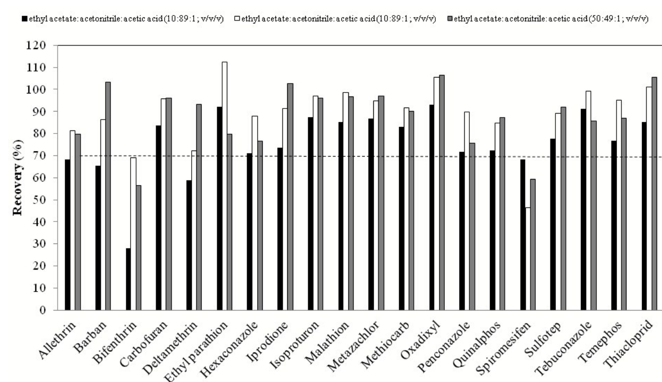

Figure 3. Recovery range of analytes allethrin, barban, bifenthrin, carbofuran, deltamethrin, spiromesifen, hexaconazole, iprodione, isoproturon, malathion, metazachlor, methiocarb, oxadixyl, ethyl parathion, penconazole, quinalphos, sulfotep, tebuconazole, temephos and thiacloprid using three different extraction solutions: (1) ethyl acetate: acetonitrile: acetic acid (10:89:1 v/v/v), (2) ethyl acetate: acetonitrile: acetic acid (30:69:1, v/v/v), (3) ethyl acetate: acetonitrile: acetic acid (50:49:1 v/v/v).

Validation method

The validation method was made based on the European Union SANCO/12571/2013 guidelines[17] and Manual of Analytical Quality Assurance from MAPA[18].

Selectivity of the method

The selectivity of the method was evaluated by injecting extracts of the blank peanuts and reagents followed by comparison of the chromatograms obtained with extracts of the blank fortified with the analytes. The signal absence in the chromatograms for the blank samples in the retention times of all the analytes studied, for both transitions monitored, confirmed the selectivity of the method.

Calibration curves

The MMC curves for each compound were built using blank sample extracts. For this, five concentrations levels were selected. The criteria adopted for the selection of the analytical curve levels were the signal to noise ratio and also the results of recovery studies. From this evaluation, the following concentration levels were selected for the MMC curves: 0.0075; 0.0100; 0.0250; 0.0500; 0.0750; 0.1000 mg kg^{-1}. The concentration level 0.0050 mg kg^{-1} was injected to confirm the LOD of the method. As described previously, OLS and WLS were used for homocedastic or heteroscedastic data, respectively. Over the calibration ranges selected, all the calibration curves presented significant linearity according to the Lack of Fit test and *t*-test on determination coefficients (r^2). LOD and LOQ are shown in Table 2 (Supplemental Information). It can be seen that LODs and LOQs were 0.0050 and 0.0100 mg kg^{-1}, respectively, except for oxamyl and tricyclazol (LOD = 0.0100 mg kg^{-1} and LOQ = 0.0250 mg kg^{-1}). For substances which the MRL is above the working range, the applicability of the method should be implemented through recovery experiments with spiked samples above the MRL, and followed by appropriate dilution by a dilution factor so that the concentration is located in the working range. This dilution should be incorporated into the calculation of measurement uncertainty.

Accuracy and precision

Recovery studies were performed at four distinct levels (0.010; 0.025; 0.050 and 0.100 mg kg^{-1}) by spiking six blank samples on three different days (hence, n = 18 for each level). The results (Table 2, Supplemental Information) were within the specified values with recovery rates between 70-120% for the all analytes at the monitoring levels, except for oxamyl and tricyclazol at 0.0100 mg kg^{-1} level. At this level these compounds did not show acceptable parameters for the recovery assays. The results can be better seen in Figure 4.

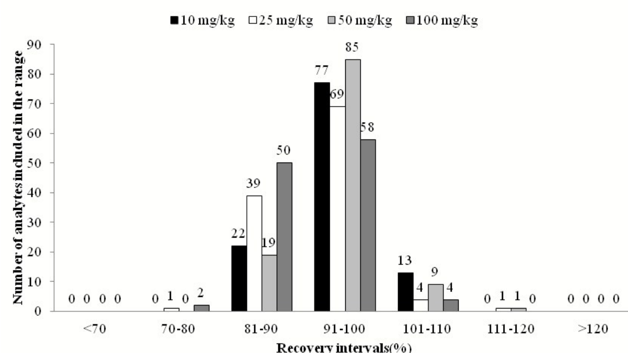

Figure 4. Recovery range of analytes validated at each level of concentration evaluated.

Intermediate precision was determined using results from two different analysts and evaluated by calculating the relative standard deviations (RSD) of the recovery rates (accuracies) for each spiking level on three different days. Independent results (n = 18) for each concentration level were achieved. Acceptable repeatability results were obtained for all the analytes at the four levels with values below 20%, except for buprofezin (23.6 %), etione (23.4 %) and picolinafen (21.0 %) at 0.100 mg kg^{-1} level. The results can be better seen in Figure 5.

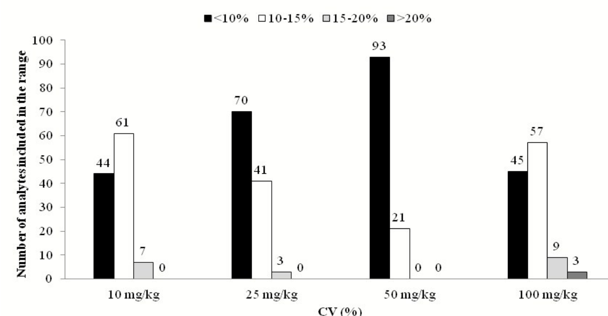

Figure 5. Intervals of coefficients of variation for the analytes validated at each level of concentration evaluated.

Measurement uncertainty

The measurement uncertainty was based on a combination of "top-down" and "bottom-up" methodologies described in EURACHEM guide [20]. The main sources of uncertainty for the method were the mass measurements of the standards for the preparation of solutions; dilution of the standard solutions; the measurements of volume of the extraction solution; the MMC curves and intermediate precision. It is noteworthy that uncertainties related to measurements of volume and mass are negligible compared to other sources raised. For all the pesticides validated, the main contribution to the total uncertainty arises from the MMC curves, because these last ones encompass all steps from the weighting of standards for preparation of solutions until the final quantification step, including the whole extraction process, the instrumental analysis and statistical

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processing of data. The expanded uncertainty (k = 2), expressed as percentage (MU %, Table 2, Supplemental Information), for each pesticide was determined in each one of the fortification levels for which the assessment of repeatability and reproducibility have been performed. The MU calculated for each pesticide presented values below 50% of the studied level, except for aldicarb sulfone (54.0 %), avermectin B1a (51.7 %), etione (52.6 %), pyraclostrobin (53.9 %), propaquizafos (52.9 %), temephos (52.8 %) and tiacloprid (54.7 %). These results agree with the acceptable criteria established in Document Nº SANCO/12571/2013[17].

Robustness

The robustness of the method was evaluated during the steps of the method optimization and validation assays. All the experiments were carried out under conditions that could influence the response such as the use of solvents of different lots as well as the environmental variations since the studies were performed on different days. However, the critical conditions of the method were kept constant. The results provided evidence that the method meets the performance criteria required, since these results were not influenced by the variability during the execution of the method. It may be concluded, therefore, that the method is robust.

Real samples

Currently, this method is being used in pesticide analysis of samples from the National Control Plan for Residues and Contaminants (PNCRC) of the Brazilian Ministry of Agriculture, Livestock and Food Supply. 37 samples of peanuts were analyzed with the validated method. No pesticide residues were found above the LOQs for most of the samples, except for one of them that presented pirimiphos methyl (0.025 mg kg^{-1}) and three that presented concentrations 0.012; 0.050 and 0.010 mg kg^{-1}, respectively.

Conclusions

In this study, a multiresidue method was successfully validated for the quantification of 113 pesticides from distinct classes in peanuts using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) techniques. This method uses a modified QuEChERS methodology based on the mixture of ethyl acetate and acetonitrile as extraction solution. A cleanup step with C-18 and PSA stationary phases was necessary due to the high amount of oil present in peanuts. The major advantages of the method described herein consist in application of a single and fast extraction/cleanup step for different analytes, i.e. polar and less polar, in fatty matrices. The overall method was validated following the requirements of Document Nº SANCO/12571/2013 [17] and the Brazilian Normative Instruction Nº SDA/MAPA 16/2013 [22]. The parameters were within the expected values with few exceptions. Thus, these promising results indicate that the present method can be applied as a routine procedure in analytical laboratories to assess pesticide residue occurrence in high fat content produce.

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Notes and references

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