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Analytical Methods

Optimization of a QuEChERS based method by means of central composite design for pesticide multiresidue determination in orange juice by UHPLC–MS/MS

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ABSTRACT

In this study, different extraction procedures based on the QuEChERS method were compared for the multiresidue determination of pesticides in orange juice by ultra high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS). After choosing preliminary conditions, an experimental design was carried out with the variables C18, PSA, NaOH and CH_3COONa to optimize the sample preparation step. The validation results of the validation were satisfactory, since the method presented recoveries between 70% and 118%, with RSD lower than 19% for spike levels between 10 and 100 μg L^{-1}. The method limit of detection (LOD) and limit of quantification (LOQ) ranged from 3.0 to 7.6 μg L^{-1} and from 4.9 to 26 μg L^{-1}, respectively. The method developed was adequate for the determination of 74 pesticide residues in orange juice.

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1. Introduction

The current use of pesticides has provided unquestionable improvements in production yield (Aktar, Sengupta, & Chowdhury, 2009). These compounds comprise a large number of substances, with different persistence levels, which are divided into different classes (herbicides, fungicides, insecticides, etc). However, the improper application of pesticides may leave residues in food, which has led different governments and international agencies to set maximum residue limits (MRLs) for food, normally at μg kg^{-1} (ANVISA, 2015). For processed food, such as juices, most often there are no MRLs established. In this context, the MRL applied to juice corresponds to the MRL for raw agricultural crops (Ravelo-Pérez, Hernández-Borges, & Rodríguez-Delgado, 2008). Brazil is the largest orange juice producer and exporter in the world (MAPA, 2014). In 2012, the exportation of orange juice from Brazil to the United States was barred due to the presence of carbendazim (fungicide) residues (EPA, 2014). In 2014, Brazil exported 1.0 million tons of orange juice, corresponding to US$ 1.9 billion (CITRUSBR, 2015), demonstrating the importance of this commodity for the Brazilian economy.

Currently, chromatographic techniques coupled with mass spectrometric detectors are the best choice for pesticide residue determination at low levels (Lacina, Urbanova, Poustka, & Hajslova, 2010; Queiroz, Ferracini, & Rosa, 2012). The LC-triple quadrupole (QqQ) mass spectrometer is one of the most popular instruments for food-quality and safety analysis, because it offers high sensitivity, selectivity and specificity for identification (Wang, Wang, & Cai, 2013). In this context, it is important to highlight the use of selected reaction monitoring (SRM) mode on detection of pesticide residues and other chemical contaminants in foods. In recent years, ultra high performance liquid chromatography (UHPLC) has shown a variety of advantages compared to traditional high performance liquid chromatography (HPLC). The combination of UHPLC with tandem mass spectrometry (MS/MS) allows high selectivity, sensitivity with reduced chromatographic run time (Fernandes, Domingues, Mateus, & Delerue-Matos, 2011).

The determination of pesticide residues in food matrices is a challenge especially because of the low concentration of analytes and large amounts of interfering substances which can be co-extracted with analytes and, in most of the cases, adversely affect the analysis results (Wilkowska & Biziuk, 2011). Several sample preparation procedures have been proposed since the 90s for pesticide residues determination in fruits and fruit juices, including: solid phase extraction (SPE), Solid Phase Micro Extraction (SPME), Matrix Solid Phase Dispersion (MSPD), Pressurized Liquid

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Extraction (PLE) and Microwave Assisted Extraction (MAE) (Fernandes et al., 2011; Prestes, Adaime, & Zanella, 2011). While these methods are highly efficient, they generally require considerable investment in instrumentation and allow a limited scope of pesticides that can be extracted under certain conditions. In this context, although they can be employed in some applications, they are far from ideal for multiresidue pesticide determinations in food, due to the wide variety of pesticides with different chemical properties (Fernandes et al., 2011; Prestes, Friggi, Adaime, & Zanella, 2009; Wilkowska and Biziuk, 2011). Since 2003, the QuEChERS method, introduced by Anastassiades, Lehotay, Štajnbaher, and Schenck (2003), there has been a revolution in sample preparation step for pesticide residue analysis. This method is based on an extraction with acetonitrile and partitioning with salt addition (Anastassiades et al., 2003). The clean-up step with a dispersive solid phase extraction (d-SPE) promotes cleaner extracts. The main sorbent used in d-SPE is a primary–secondary amine (PSA) which provides high capacity for the removal of sugars, organic and fatty acids and polar pigments. C18 silica sorbent is used in fat-containing samples. The Graphitized Carbon Black (GCB) sorbent can be added for the clean-up of highly pigmented samples (Wilkowska and Biziuk, 2011). This method was proposed in order to overcome the limitations of the methods mentioned above. Moreover, it supplies the necessary characteristics for a multiresidue method and thus ensures accurate and precise results and low limits of detection for a large range of compounds (Anastassiades et al., 2003). In recent years, this method was validated for different food matrices: vegetables (Du et al., 2013); fruits (Sousa et al., 2013); meat (Liu et al., 2014); cereals (Hou et al., 2013); vegetable juice (Nguyen, Yun, & Lee, 2009); fruit juices (Romero-González, Frenich, & Vidal, 2008; Tran et al., 2012).

Therefore, considering the importance of orange juice for Brazilian economy this study aims to develop and validate a rapid and effective method for the determination of pesticide residues in orange juice. We highlight in this paper the use of central composite design for the optimization of QuEChERS method. UHPLC–MS/MS method was developed based on selected reaction monitoring (SRM) for the determination of 74 compounds.

2. Materials and methods

2.1. Chemicals and reagents

Certified pesticide standards were purchased from Dr. Ehrenstorfer (Augsburg, Germany), with purity between 81.0% and 99.9%. The studied pesticides were selected taking into account their application in orange groves in Brazil. Individual standard solutions of each pesticide were prepared at a concentration of 1000 mg L^{-1} in acetonitrile. Standard mixed solutions (5 mg L^{-1} and then 1 mg L^{-1}) were prepared by mixing suitable volumes of individual standard solutions and diluting with acetonitrile. These mixtures were stored in amber flasks at ±2 °C.

Analytical-grade ammonium acetate (CH_3COONH_4), sodium hydrogencitrate sesquihydrate ($C_6H_6Na_2O_7{\cdot}1.5H_2O$) and sodium citrate tribasic dihydrate ($C_6H_5Na_3O_7{\cdot}2H_2O$) were purchased from Sigma Aldrich (St. Louis, USA). The salts, sodium chloride, sodium acetate and anhydrous magnesium sulfate (J.T. Baker, Ecatepec de Morelos, México), used in this study were laboratory reagent grade. Acetonitrile (MeCN), pesticide residue grade, and acetic acid (HAc) analytical grade were purchased from J.T. Baker (Phillipsburg, USA). The sorbents, primary secondary amine (PSA) and octadecyl modified silica (C18) were supplied by Agilent Technologies (Santa Clara, USA). Water was ultra-purified in a MilliQ® system from Millipore (Molsheim, France) and was used for the preparation of buffer solutions and mobile phase.

2.2. Instrumentation

Vortex mixer model QL-901 Microtecnica (Curitiba, Brazil); analytical balance, model UX-420H from Shimadzu (Kyoto, Japan); refrigerated centrifuge NT 825 NovaTecnica (Piracicaba, Brazil); SL703 refrigerated centrifuge Solab (Piracicaba, Brazil); automatic micropipettes with variable capacity from Brand (Wertheim, Germany); and juice processor (Arno, São Paulo, Brazil) were employed.

Chromatographic analyses were performed using UHPLC–MS/MS system (Waters, Milford, USA) equipped with Acquity UPLC™ liquid chromatography; Xevo TQ™ MS/MS triple quadrupole detector, an autosampler, a binary pump and a column temperature controller; A Waters (Wexford, Ireland) Acquity UPLC™ BEH C18 (50 × 2.1 mm i.d., 1.7 μm particle size) analytical column was used for UHPLC separations. Column oven temperature was 40 °C. MassLynx 4.1 software (Waters, Milford, USA) was used for instrument control and data processing. The mobile phase consisted of (A) water:methanol (98:2, v/v), and (B) methanol, both containing 0.1% formic acid and 5 mmol L^{-1} ammonium formate. These solutions were sonicated (30 min) before use. The gradient program started at 5% B (held 0.25 min) increased to reach 100% B in 7.75 min (held 0.75 min) and decreased to reach 5% B in 8.51 min (held 1.49 min). The optimum flow rate was 0.25 mL min^{-1} whereas the injection volume was 10 μL. The total chromatographic run time was performed in 10 min.

To quantify the pesticides, mass spectrometer was operated in the electrospray ionization positive mode (ESI+) using SRM. The MS source conditions were as follows: capillary voltage, 2.0 kV; source temperature, 150 °C; desolvation temperature, 500 °C; desolvation gas (N_2) flow, 600 L h^{-1}; and cone gas (N_2) flow, 80 L h^{-1}. Each pesticide solution was primarily infused directly in the MS and the mass spectra with the $(M+H)^+$ ion was obtained using the quadrupole 1 (Q1) in positive mode. Specific SRM transitions with Q3 were implemented using the above information regarding Q1 to permit identification of the target pesticides through the selection for each compound. Collision-induced dissociation was performed using argon as collision gas with flow rate at 0.15 mL min^{-1}. For precursor and product ions identification in positive mode, preliminary optimization of the instrument settings was performed by continuous infusion of the individual compound at 500 μg L^{-1} in acetonitrile. The maximum tolerance for ion ratios was ±30%, as recommended by SANCO/12571/2013. The cone voltage, collision energy parameters, ion ratio and SRM transition for each pesticide analyzed are shown in Table S-1 (Supplementary data).

2.3. Sample processing

Orange samples of 1 kg each were obtained from supermarkets in Santa Maria (Brazil) in January 2014. In total, the juice from 14 orange samples were analyzed. The oranges were processed in a juicer in order to make the orange juice extracting process as close as possible to that used at home. Average yield of orange juice for each sample was around 500 mL. The juice samples were maintained in polypropylene (PP) bottles, properly identified and, if necessary, stored in a freezer at <−18 °C.

2.4. Optimization of Sample Preparation by experimental design

2.4.1. Preliminary tests

Several procedures based on QuEChERS method were evaluated in order to establish the best extraction. Aliquots of 10 mL of blank sample spiked at 20 μg L^{-1} were transferred to a 50 mL PP centrifuge tube and extracted as follow:

2.4.1.1. Method A (QuEChERS original). 10.0 mL of acetonitrile were added and the tubes were shaken during 1 min. Then, anhydrous magnesium sulfate (4.0 g) and sodium chloride (1.0 g) were added.

2.4.1.2. Method B (QuEChERS citrate). 10.0 mL of acetonitrile were added, and the tubes were shaken during 1 min. Then, anhydrous $MgSO_4$ (4.0 g), NaCl (1.0 g), tri-sodium citrate dihydrate (1.0 g) and di-sodium hydrogencitrate sesquihydrate (0.5 g) were added.

2.4.1.3. Method C (QuEChERS acetate). 10.0 mL of 1% (v/v) acetic acid in acetonitrile were added, and the tubes were shaken during 1 min. Next, anhydrous $MgSO_4$ (4.0 g) and CH_3COONa (1.7 g) were added.

2.4.1.4. Method D (QuEChERS ammonium acetate). 10.0 mL of 1% (v/v) acetic acid in acetonitrile were added, and the tubes were shaken during 1 min. Next, anhydrous anhydrous $MgSO_4$ (4.0 g) and CH_3COONH_4 (1.7 g) were added.

For all methods, after the salt addition, the tubes were shaken during 1 min and immediately centrifuged for 8 min at 2420*g*. Then, 1 mL of the supernatant was transferred into a 2 mL Eppendorf tube for clean-up step by two procedures:

Clean-up 1: 50 mg of PSA, 50 mg C18 and 150 mg of anhydrous $MgSO_4$.
Clean-up 2: 50 mg of PSA and 150 mg of anhydrous $MgSO_4$.

In both clean-up procedures the mixture was shaken for 1 min and centrifuged for 3 min at 13,316*g*. Then, the supernatant was filtered through a Millex-GN nylon filter 0.20 μm (Millipore, Carrigtwohill, Ireland) and diluted 5× with water prior to UHPLC–MS/MS analysis.

2.4.2. Central composite design (CCD)

From the results obtained in preliminary tests, an optimization using central composite design (CCD) (Nasirizadeh, Dehghanizadeh, Yazdanshenas, Moghadam, & Karimi, 2012) was performed. The CCD evaluation with four factors was used to determine the optimal conditions for QuEChERS method. Four independent variables were selected: sodium hydroxide (X_1), C18 (X_2), PSA (X_3) and sodium acetate (X_4). Each variable was investigated at five levels: $-\alpha$, -1, 0, +1 and $+\alpha$, as shown in Table 1. The center point was evaluated in triplicate. Thus, a total of 27 different combinations of random order were performed according to a CCD configuration for 4 factors. The quantities of orange juice (10 mL), acidified MeCN (10 mL) and $MgSO_4$ (4 g) remained constant.

2.4.2.1. Proposed and validated method based on QuEChERS acetate procedure. 10 mL of blank sample were transferred to a 50 mL PP centrifuge tube. Then 10.0 mL of 1% (v/v) acetic acid in acetonitrile was added, and the tubes were shaken during 1 min. Next, $MgSO_4$ (4.0 g) and sodium acetate (1.7 g) were added and the tubes were shaken during 1 min and immediately centrifuged for 8 min at 2420*g*. For the clean-up step 1.0 mL of the supernatant was transferred into a 2 mL Eppendorf tube containing 40 mg of PSA and 150 mg of anhydrous $MgSO_4$. The mixture was shaken for 1 min and centrifuged for 3 min at 13,316*g*. Then, the supernatant was filtered through a 0.20 μm filter and diluted 5 times with water prior to UHPLC–MS/MS analysis.

Table 1
Central composite design with the four independent variables evaluated.

	Levels				
	$-\alpha$ (−2)	Low (−1)	Center (0)	High (+1)	$+\alpha$ (+2)
(X_1) NaOH (μL)	0	50	100	150	200
(X_2) C18 (mg)	0	20	40	60	80
(X_3) PSA (mg)	0	20	40	60	80
(X_4) CH_3COONa (g)	0.3	1.0	1.7	2.4	3.1

2.5. Method validation

In this study, validation data sets were carried out according to the European SANCO/12571/2013 guidelines (SANCO, 2013). Selectivity, matrix effect, linearity and working range, limit of detection (LOD), limit of quantification (LOQ), accuracy and precision were evaluated. Selectivity was evaluated by extraction and analysis of a "blank" sample and also extraction only with the reagents, called "reagent blank". The matrix effect was studied through the comparison between calibration curves prepared in solvent and matrix-matched. Linearity was assessed using curves at concentration levels of 0.5, 1.0, 2.0, 5.0, 20.0, 50.0, 100.0 and 200.0 μg L^{-1}. Accuracy, in terms of recovery, and precision, expressed in terms of repeatability (RSD), were evaluated with orange juice blank samples spiked at 10, 50 and 100 μg L^{-1} after analyzing seven replicates at each concentration. Inter-day precision was evaluated at 50 μg L^{-1} and seven spiked samples were analyzed in different days.

Method LOQ values were obtained experimentally from orange juice blanks spiked at 10.0 μg L^{-1} ($n = 7$). The LOQ determination was based on pre-defined acceptance criteria of recovery from 70% to 120% and RSD <20% (SANCO, 2013). In cases that this spike level did not achieved the required results of recovery and RSD, an additional spike level of 25 μg L^{-1} was employed. Recoveries values were evaluated by the Grubbs test in order to verify outliers. Then, considering the lower level that provided adequate results, the LOQ values were calculated as $LOQ = m - \frac{t \cdot s}{\sqrt{n}}$, where m is the average of the results, t is the Student's value for two-tailed distribution with a confidence level of 99%, s is the sample standard deviation and n number of replicates (INMETRO, 2011; Shrivastava & Gupta, 2011). Method LOD values were obtained dividing by 3.3 the lower spiked level that provided adequate results of recovery and RSD.

Triphenylphosphate (TPP) and deuterated linuron were used as the internal standard and surrogate, respectively. The proposed method was applied to orange juice samples from Santa Maria-RS (Brazil). All samples were analyzed following the procedure validated and samples without residues of the target compounds were used as blank samples in the preparation of matrix matched standards and for recovery studies.

3. Results and discussion

3.1. Optimization of the UHPLC–MS/MS system

Mobile phase modifiers were used to improve analyte signals and to obtain better reproducibility for chromatographic responses. With ammonium formate, the presence of ammonium ions suppressed the formation of sodium adducts, which are more common under acidic conditions (formic acid), and therefore, pesticides formed predominantly $[M+H]^+$ and $[M+NH_4]^+$, which showed higher sensitivity and more consistent responses for certain pesticides (Hiemstra & Kok, 2007). Table S-1 shows the optimized transitions, collision energies and retention time for all compounds analyzed.

For the UHPLC–MS/MS, the most abundant ion was selected as the precursor ion and then it was isolated in the first quadrupole. Different collision energies were applied and promoted ion fragmentations to obtain the corresponding product ions. From the product ion, two transitions were selected for each pesticide to

work under SRM mode. Matrix-matched standard chromatogram at 20 μg L^{-1} is shown in Fig. S-1 (Supplementary data).

3.2. Investigation of the modified QuEChERS method

Orange juice contains carbohydrates, proteins, fatty acids, vitamins, minerals, and water as the major contents. Many of these components are antioxidants which can result in pesticides slow degradation. Thus, different modifications of QuEChERS methods were evaluated in terms of ability to provide good analyte extraction from a sample with minimal extraction of undesirable interfering components.

In this study, the choice of acidified acetonitrile is to provide extraction of a wide range of pesticides with different polarities. The use of $MgSO_4$ in the partition step reduces the volume of aqueous phase by hydration and saturates the molecular spaces within the aqueous solvent, resulting in a solubility decrease of polar analyses. The addition of salt, such as sodium or ammonium acetate (Frenich, Vidal, Pastor-Montoro, & Romero-González, 2008), besides helping to promote the salting out effect, allows, together with the 1% (v/v) acetic acid added in acetonitrile, the buffering of the medium. This enables a better extraction of pesticides that usually have stability problems. Based on the interferences expected in orange juice samples, PSA and C18 sorbents were selected. Due to the bidentate structure, PSA has a high chelating effect. As a result of the secondary and primary amino groups the retention of free fatty acids and other polar matrix compounds is very strong (Plössl, Giera, & Bracher, 2006). The C18 sorbent is more hydrophobic with an extremely retentive nature for non-polar compounds, such as fat (Plössl et al., 2006).

A total of 76 compounds were analyzed by different methods. Recoveries between 70% and 120% and RSD <20% were adopted as satisfactory results The percentage of compounds with acceptable results for each test was 70 (method A + clean-up 1), 77 (method A + clean-up 2), 75 (method B + clean-up 1), 80 (method B + clean-up 2), 86 (method C + clean-up 1), 92 (method C + clean-up 2), 89 (method D + clean-up 1) and 92 (method D + clean-up 2). With the use of acetate and citrate buffers the pH of the extract gets close to 4.8 and 5.0–5.5, respectively, facilitating the extraction of low-pH susceptible compounds, such as thiabendazole and imazalil and/or those that present stability problems (Prestes et al., 2009). The tests that used ammonium acetate (method D) and sodium acetate (method C) buffer resulted in a higher number of compounds with satisfactory accuracy and precision. This was expected, because orange juice is a matrix with pH close to 3.5, which makes it difficult to extract some pesticides without pH adjustment. The use of ammonium acetate showed a slight improvement in the sensitivity of the signal. However, considering there were comparable results for both buffers, the QuEChERS buffered with sodium acetate (method C) was chosen. In the clean-up step when C18 and PSA were used together there was a decrease in the recovery of some compounds. This occurred due to the larger amount of sorbent used and possible retention of the non-polar compounds in the sorbent C18. Although the tests indicate that the clean-up with PSA showed better recoveries, we opted for the choice of PSA and C18 (clean-up 1) for optimization by CCD, since their combination often provides more effective clean-up. Thus, Method C + clean-up 1 was selected for optimization of the sample preparation.

3.3. Central composite design for the optimization of the sample preparation step

Once the best method of extraction was chosen, an optimization of sample preparation was performed through CCD. Considering previous results, the use of the extraction medium buffered with sodium acetate improved the efficiency of pesticide extraction; hence different amounts of this salt were tested. According to published studies for acid rich samples, the pH value achieved after the addition of buffering salt is usually lower than expected and thus in order to protect acid-labile compounds the pH-value can be elevated by adding 5 mol L^{-1} of NaOH, such as in the case of lemons (Kittlaus, Schimanke, Kempe, & Speer, 2012). Therefore the NaOH variable was chosen to verify its effect on the compounds recovery. In preliminary tests, variations of the sorbents PSA and C18 were not tested and thus it was not clear what effect their use had on the clean-up step and so these variables were included. The variables were chosen to evaluate the extraction procedure and also the clean-up step.

The Pareto chart of standardized effects was used to demonstrate the effect of two way interactions. The effects considered significant are those that are outside the range of the estimated error, and thus influence positively or negatively the obtained results in the number of compounds with acceptable recovery (70–120%). As can be seen in Fig. 1, C18, NaOH and interaction of PSA by CH_3COONa showed negative effect; the interaction of NaOH by C18 and PSA was positive effect. For other interactions and variable are not considered significant. Therefore, it can be concluded that with the use of only PSA the positive effect is more evident.

Considering these results the parameters NaOH (X_1), C18 (X_2), PSA (X_3) and CH_3COONa (X_4) were investigated by response surface methodology. Fig. 2a demonstrates that using intermediate amounts (1.7 g) of sodium acetate and 40 mg PSA good recovery results were obtained without the addition of C18 and NaOH, while Fig. 2b reveals that using a maximum amount (80 mg) of PSA and intermediate amounts (1.7 g) of sodium acetate the addition of C18 is not necessary and the amount of NaOH added is the minimum possible. Based on these results it can be stated that: (1) the intermediate amount of acetate is adequate (2) there is no need to use C18 in the clean-up step and (3) to employ PSA alone is sufficient. As shown in the previous tests the use C 18 was not effective for orange juice. Thus, Fig. 2a shows the best conditions, due to the lower amount of sorbent required. Fig. 2b indicates better recoveries with low amounts of NaOH and maximum PSA. The use of NaOH represent one more step in the extraction process and the use of large amounts of PSA increases the analysis costs, considering that 40 mg was enough to obtain good recoveries. Thus, the validated method was established without NaOH addition and with 40 mg of PSA how shown in Fig. S-2 (Supplementary data).

In 2007 the method "QuEChERS acetate" was considered the official method (2007.01) of the Association of Official Analytical Chemists for the determination of pesticide residues in food. The quantities of sample and reagents used in the official method are 15 g of sample, 15 mL of acidified MeCN, 6 g $MgSO_4$, 1.5 g of CH_3COONa for the extraction step and 150 mg of $MgSO_4$ and 50 mg of PSA to 1 mL of the supernatant for the clean-up step. When compared with the official method the procedure validated in this work differs by the reduction in the amount of sample, acidified MeCN, $MgSO_4$ and PSA used. The amount of sodium acetate used was slightly higher than that employed in the official method. This was necessary, possibly due to the high acidity of orange juice, since a lower amount of this salt may not result in adequate pH buffering.

3.4. Validation of the method

Method selectivity was guaranteed, since there were no interferences for quantification and confirmation ions at the retention time of the analyte in the blank sample studied. Thus, the blank sample was used to prepare the calibration curves and spiking. Solvents and reagents were also evaluated, using the extraction procedure, however without the sample. This also verified the absence of contaminants that might interfere in the analysis.

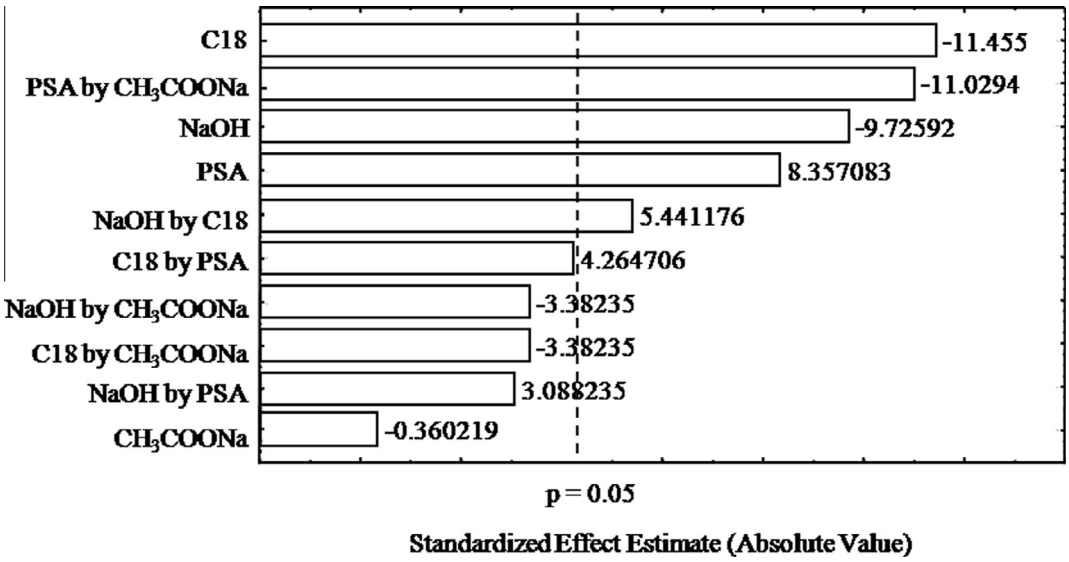

Fig. 1. Pareto chart of standardized effects.

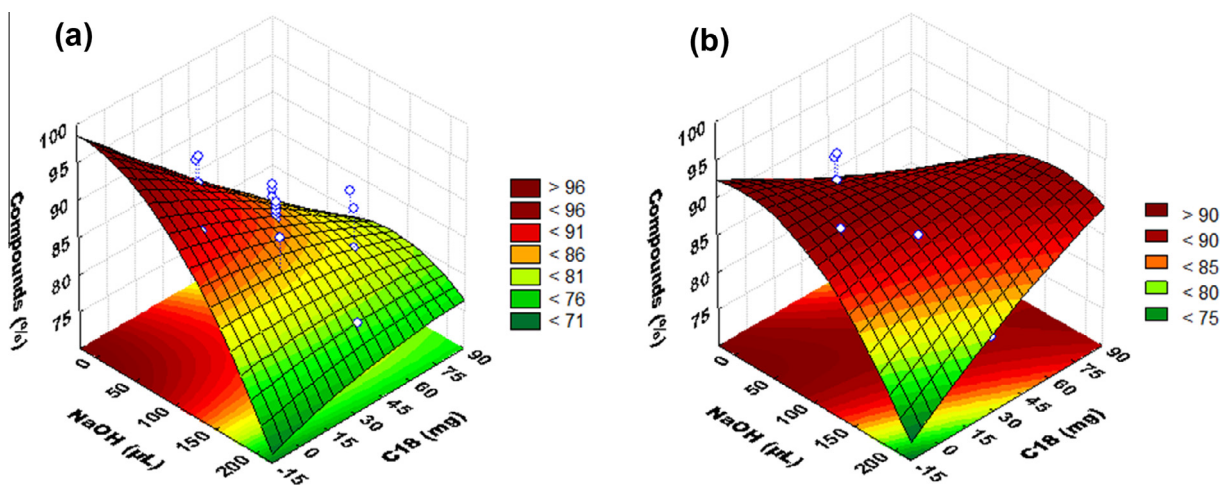

Fig. 2. Response surface generated by the experimental design considering the compounds with acceptable recovery using intermediate condition of sodium acetate (1.7 g) and PSA (a) 40 mg and (b) 80 mg.

Based on the results obtained from standard curves, it can be concluded that the linear and quadratic equations are satisfactory. Given that, most of the compounds showed coefficients of determination (r^2) $\geqslant$ 0.99 which are adequate for residue analysis. It is noteworthy that for those compounds which did not present $r^2 \geqslant 0.99$ with the linear model, the quadratic model was suitable. The use of the TPP, as internal standard, was used in order to check the stability of the instrument through the reproducibility of the chromatographic signal in all injections and proved to be effective for this purpose.

Of the compounds studied only acephate and deltamethrin were not recovered in any test. This may be because these compounds require different conditions of analysis. Acephate degrades easily and thus requires special conditions, such as the use of low temperatures of the source and/or desolvation. This is also a problem that occurs when using GC–MS. Deltamethrin is a pyrethroid and is more suitable for quantification by GC–MS compared to LC–MS (Raina, 2011). For benomyl no chromatographic signal was obtained due its quick degradation in solvent. Several studies have reported that the compound benomyl is very unstable and degrades easily. Thus, the presence of carbendazim as an impurity in benomyl standards and the high degradation rate of benomyl in organic solvents make it practically impossible to obtain carbendazim-free solutions. This degradation is the result of hydrolysis, in which the benomyl loses an amide group and degrades into carbendazim (Fig. S-3, Supplementary data) (Anastassiades & Schwack, 1998). So, the compound benomyl is quantified in the form of carbendazim.

Matrix effects are known to be problematic with regard to the analysis of pesticide residues by LC–MS/MS resulting in increase or suppression of the analytic signal (Hajšlová & Zrostlíková, 2003). There are several factors that can interfere with ionization, identification and quantification in ESI interfaces, and therefore the appropriate sample preparation and optimization of analysis conditions are of fundamental importance. In this study, for most compounds, the matrix effect was not considered significant because the values were in the range of ±20% (Ferrer, Lozano, Agüera, Girón, & Fernández-Alba, 2011). However, for some pesticides such as carbofuran, the matrix effect showed higher intensity in the presence of the matrix. For this reason, curves were prepared in the matrix extract for the quantification of the analytes. Before the chromatographic analysis, the extracts were diluted 5 times

Table 2
Recoveries and RSD of spiked levels studied for intra and inter-day, method limit of quantification (LOQ) and limit of detection (LOD), and matrix effect.

Compounds	Rec (RSD)% for different spike levels (μg L^{-1})				LOQ (μg L^{-1})	LOD (μg L^{-1})	Matrix effect (%)
	Intra-day[b]			Inter-day[c]			
	10	50	100	50			
3-OH-carbofuran	106 (12)	91 (9)	75 (10)	87 (15)	8.0	3.0	34
Acetamiprid	99 (9)	91 (7)	80 (7)	88 (9)	8.7	3.0	2
Ametryn	84 (13)	93 (6)	80 (6)	99 (1)	6.8	3.0	6
Atrazine	97 (10)	92 (11)	79 (8)	93 (2)	8.1	3.0	5
Azoxystrobin	94 (11)	86 (7)	76 (9)	86 (8)	7.8	3.0	5
Boscalid	107 (13)	89 (11)	80 (14)	93 (12)	8.3	3.0	2
Bromuconazole	89 (16)	78 (13)	81 (11)	85 (16)	5.9	3.0	4
Buprofezin	101 (10)	86 (7)	80 (6)	98 (7)	6.2	3.0	50
Carbaryl	100 (11)	91 (5)	83 (7)	88 (10)	7.7	3.0	45
Carbendazim	99 (10)	84 (5)	77 (3)	88 (5)	7.6	3.0	−1
Carbofuran	91 (10)	88 (2)	75 (7)	91 (4)	7.5	3.0	77
Carboxin	88 (13)	84 (4)	82 (10)	86 (10)	7.2	3.0	5
Cyanazine	106 (16)[a]	100 (11)	88 (7)	99 (5)	19.3	7.6	5
Clomazone	105 (6)	88 (12)	89 (9)	95 (10)	7.4	3.0	2
Demeton-S-methyl-sulfone	79 (11)	84 (9)	77 (4)	81 (6)	5.7	3.0	44
Diazinon	93 (8)	73 (15)	83 (3)	87 (10)	6.7	3.0	−2
Dicrotophos	87 (16)	88 (14)	74 (8)	86 (14)	5.8	3.0	−1
Difenoconazole	111 (12)	75 (16)	102 (9)	97 (19)	6.2	3.0	−3
Dimethoate	101 (10)	87 (14)	83 (10)	87 (12)	8.6	3.0	5
Dimethomorph	104 (9)	86 (11)	72 (3)	84 (6)	8.5	3.0	1
Dimoxystrobin	112 (19)	97 (11)	82 (8)	95 (10)	6.9	3.0	−1
Diuron	101 (11)	91 (4)	77 (3)	87 (9)	7.7	3.0	5
Dodemorph	110 (11)	91 (2)	73 (7)	91 (8)	9.3	3.0	39
Epoxiconazole	90 (18)[a]	82 (12)	89 (8)	109 (6)	14.0	7.6	−10
Ethiofencarb sulfone	99 (7)[a]	93 (7)	79 (6)	96 (7)	22.4	7.6	97
Ethiofencarb sulfoxide	90 (17)[a]	94 (7)	78 (4)	95 (5)	16.0	7.6	111
Etoprophos	82 (17)	73 (12)	83 (10)	85 (9)	5.4	3.0	1
Fenpyroximate	116 (6)[a]	75 (12)	97 (17)	108 (10)	25.5	7.6	−10
Fenpropimorph	92 (9)	86 (1)	86 (8)	81 (7)	7.5	3.0	39
Fenamiphos	87 (8)	83 (7)	83 (8)	86 (4)	7.3	3.0	38
Fenazaquin	118 (6)[a]	75 (7)	91 (2)	115 (5)	26.0	7.6	16
Fluazifop-*p*-butyl	91 (13)	70 (17)	76 (7)	89 (9)	7.1	3.0	7
Flusilazole	110 (4)[a]	103 (8)	92 (10)	99 (13)	25.4	7.6	2
Flutolanil	92 (14)	77 (16)	93 (14)	76 (12)	6.5	3.0	1
Flutriafol	111 (11)[a]	92 (16)	82 (10)	104 (15)	23.4	7.6	−1
Imazalil	103 (13)	77 (6)	79 (8)	76 (5)	8.5	3.0	−8
Linuron	90 (16)	87 (4)	83 (5)	87 (7)	6.0	3.0	−10
Linuron deuterated	88 (6)	88 (7)	87 (5)	84 (10)	6.0	3.0	1
Mecarbam	101 (18)[a]	87 (9)	88 (18)	103 (16)	15.9	7.6	15
Mephosfolan	109 (6)	89 (2)	84 (6)	89 (4)	10.0	3.0	−1
Mepronil	90 (13)	81 (4)	89 (9)	89 (10)	7.4	3.0	−8
Metalaxyl	107 (12)	95 (8)	74 (8)	92 (7)	8.8	3.0	3
Methiocarbe sulfone	92 (12)	80 (8)	82 (5)	84 (10)	6.4	3.0	90
Metobromuron	96 (14)	90 (6)	75 (3)	90 (4)	7.3	3.0	−1
Metolachlor	110 (11)	101(6)	81 (10)	101 (9)	8.6	3.0	3
Mevinphos	99 (12)	85 (5)	84 (6)	86 (10)	8.2	3.0	3
Monocrotophos	97 (14)[a]	83 (9)	73 (6)	83 (13)	18.5	7.6	5
Monolinuron	97 (16)	85 (3)	81 (8)	89 (3)	7.6	3.0	0
Omethoate	104 (9)	76 (7)	71 (7)	76 (4)	9.1	3.0	34
Paraoxon-ethyl	89 (12)	79 (6)	82 (12)	89 (8)	7.4	3.0	1
Penconazole	94 (16)	78 (12)	91 (9)	94 (8)	7.0	3.0	−4
Picoxystrobin	84 (14)	87 (13)	82 (7)	90 (10)	5.9	3.0	−2
Pyridaphenthion	111 (14)[a]	106 (7)	82 (12)	105 (11)	22.2	7.6	3
Pyrifenox	117 (15)[a]	105 (8)	92 (10)	112 (12)	21.8	7.6	5
Pirimiphos-methyl	104 (17)	76 (17)	80 (5)	89 (8)	7.9	3.0	3
Pyriproxyfen	87 (15)[a]	76 (10)	83 (7)	102 (9)	17.2	7.6	44
Propanil	88 (14)	75 (15)	78 (7)	82 (10)	6.3	3.0	6
Propiconazole	93 (6)	78 (8)	88 (5)	76 (14)	8.3	3.0	−4
Rotenone	97 (13)	70 (18)	78 (16)	97 (16)	7.6	3.0	−1
Spinosad A	87 (18)[a]	90 (16)	93 (14)	86 (17)	16.2	7.6	29
Simazine	82 (16)	74 (10)	76 (8)	80 (9)	6.0	3.0	−11
Tebuconazole	85 (20)[a]	96 (17)	82 (5)	95 (14)	15.3	7.6	−1
Tebufenozide	117 (10)	98 (10)	90 (9)	95 (8)	9.3	3.0	−1
Terbuthylazine	78 (19)	86 (9)	85 (13)	99 (7)	5.3	3.0	6
Tetraconazole	100 (19)	78 (14)	80 (18)	71 (12)	6.9	3.0	1
Thiabendazole	86 (11)	83 (7)	80 (3)	83 (7)	7.3	3.0	4
Thiacloprid	101 (9)[a]	96 (12)	85 (10)	91 (11)	22.1	7.6	−10
Thiobencarb	81 (19)	86 (12)	84 (5)	96 (12)	4.9	3.0	3
Thiophanate-methyl	77 (15)[a]	81 (6)	85 (5)	82 (15)	13.1	7.6	7
Triadimefon	98 (7)[a]	91 (11)	91 (6)	93 (13)	21.2	7.6	1
Triazophos	113 (9)	88 (7)	92 (9)	82 (8)	9.7	3.0	4

Table 2 (*continued*)

Compounds	Rec (RSD)% for different spike levels (μg L^{-1})				LOQ (μg L^{-1})	LOD (μg L^{-1})	Matrix effect (%)
	Intra-day[b]			Inter-day[c]			
	10	50	100	50			
Triflumizole	89 (12)	97 (5)	81 (7)	99 (8)	7.1	3.0	28
Triflumuron	94 (19)	78 (7)	79 (10)	85 (16)	7.0	3.0	−5
Vamidation	98 (18)	99 (13)	90 (9)	86 (11)	6.1	3.0	4

[a] Values referring to the level of 25 μg L^{-1}.
[b] Intra-day repeatability was estimated by analyzing seven replicate samples at three concentration levels on the same day.
[c] Inter-day repeatability was estimated by analyzing seven replicate samples in different days.

Table 3
Results of the analyses in real samples and the maximum residues level (MRL) from different organizations.

Sample number	Concentration of pesticide residues (μg L^{-1})					
	Buprofezin	Carbendazim	Pirimiphos-methyl	Triazophos	Paraoxon-ethyl	Simazine
2	nd	9.4	nd	nd	nd	<LOQ
3	nd	10.0	nd	nd	<LOQ	nd
6	nd	28.8	nd	nd	<LOQ	<LOQ
7	nd	9.6	nd	nd	<LOQ	nd
8	20.4	16.0	16.1	nd	<LOQ	nd
9	nd	9.8	nd	<LOQ	<LOQ	nd
10	nd	26.1	nd	nd	nd	nd
11	nd	nd	nd	nd	<LOQ	nd
13	nd	9.2	15.4	nd	nd	<LOQ
14	nd	10.1	nd	nd	nd	<LOQ
	MRL (μg kg^{-1})					
ANVISA	300	5000	5000	10	NE	20
USEPA	2500	NE	NE	NE	250	NE
EU	1000	200	1000	10	20	10

MRL – maximum residue limit; nd = no detected; NE – not established; ANVISA, Brazil (2014); EU – European Union (EU) (2013); USEPA – United States Environmental Protection Agency (2013).

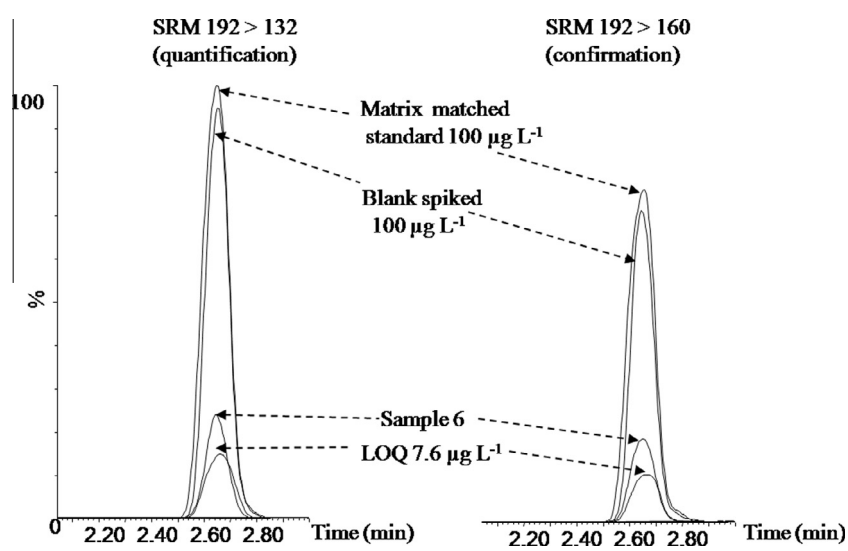

Fig. 3. Comparison of the UHPLC–MS/MS signals of carbendazim quantified in sample 6, with spiked and matrix matched standard at the concentration of 100 μg L^{-1}.

with water in order to reduce matrix effect and the amount of matrix injected, as well to have better peak shapes. The values for matrix effect are presented in Table 2. Fig. S-4 (Supplementary data), shows the comparison of matrix-matched and in solvent calibration curves for matrix effect, clearly demonstrating the importance of the quantification of samples using matrix matched calibration.

Method LOD and LOQ ranged from 3.0 to 7.6 μg L^{-1} and from 4.9 to 26.0 μg L^{-1}, respectively. The values are presented in Table 2 and LOQs are lower than those established by law for the compounds that are allowed in orange (ANVISA, 2014), thus the method is suitable for the analysis of real samples.

Spiking levels for the recovery study were chosen taking into account the MRLs established by national legislation and recovery and precision results are presented in Table 2. All compounds showed acceptable recoveries and precision results in the range of 70–118% and RSD <19%, respectively. The intermediate precision was evaluated inter-day and presented recoveries from 71% to

115% with RSD <19%, considered satisfactory. The surrogate standard spiked at the same concentration of the analytes showed good accuracy and precision results. The calibration range was from the respective LOQ to 200 μg L^{-1}, with r^2 values between 0.9925 and 1.000.

3.5. Application to real samples

Table 3 presents the results of the analyzed positive samples. Pesticide residues of paraoxon-ethyl, simazine and triazophos were found below the LOQ. However, the compounds buprofezin, pirimiphos-methyl and carbendazim were found in concentrations above the LOQ. In samples 1, 4, 5 and 12, traces of pesticide residues triazophos, paraoxon-ethyl and simazine were found below the LOQ.

Fig. 3 compares the chromatographic signal of carbendazim presented in sample 6, with spiked and matrix matched standard at the concentration of 100 μg L^{-1}. In general, for countries in which the use of a pesticide is prohibited for certain crops the maximum limit acceptable is 10 μg kg^{-1} (EC, 2005). However, almost all the samples had residues of carbendazim at concentrations close to or above this limit. The fungicide carbendazim is widely used in crop protection. It is also the main degradation product of two other compounds: benomyl and thiophanate-methyl (Anastassiades and Schwack, 1998). They are usually applied in post-harvest on bananas, citrus fruits, pome fruits, mangoes and potatoes to protect them from decay caused by various fungal pathogens. Thus, the residues of carbendazim can be generated as the degradation product of the pesticides benomyl and thiophanate-methyl. As seen previously, benomyl degrades easily in carbendazim. However, thiophanate-methyl is more stable than benomyl and generally persists over long periods in treated crops. The determination of thiophanate-methyl together with carbendazim is a routine procedure (Anastassiades and Schwack, 1998).

4. Conclusion

The buffered QuEChERS extraction method using acidified acetonitrile and sodium acetate proved to be effective for the extraction of 74 pesticides in orange juice. The conditions of extraction and clean-up steps were optimized using CCD, which revealed that the use of sodium acetate and PSA alone is sufficient for extraction and clean-up, respectively. Unnecessary steps, such as the addition of sodium hydroxide, which would be more time consuming and susceptible to analytical errors, were avoided. The modified QuEChERS extraction method, optimized in this work, has the advantage of being a dynamic, simple and fast procedure, with few analytical steps, which minimize errors. In addition, it is cheap and environmentally benign due to the use of only 10 mL of acetonitrile.

In order to compensate the matrix effect the quantification was performed using matrix matched standards. From the studied pesticides, only acephate and deltamethrin could not be quantified in this study. The compound benomyl is very unstable and degrades easily, thus it was expressed as carbendazim. The compounds showed LOQs between 4.9 and 26 μg L^{-1}.

The use of UHPLC–MS/MS with ESI+, operating in the SRM mode provided satisfactory selectivity and high sensitivity. The proposed method proved to be an important tool for analysis of pesticide residues in orange juice and can be used in routine analysis. The application indicated that paraoxon-ethyl, simazine and triazophos presented concentrations below the LOQ and buprofezin, carbendazim and pirimiphos-methyl above the LOQ, but below the MRLs established by Brazilian legislation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.09.010.

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