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Improving analytical performance for pesticides and mycotoxins determination in Brazilian table olives: one extraction and one analysis†

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This paper describes an extensive study in which a multiclass QuEChERS based approach was optimized for determination of 150 pesticides and 7 mycotoxins in table olives. Three versions of QuEChERS were evaluated and compared (unbuffered, citrate and acetate buffering). A combination of EMR-Lipid cartridges and liquid nitrogen or freezer freezing out were tested for clean-up of the oily olive extracts. Analysis of the extracts were performed by LC-MS/MS triple quadrupole. The best results were achieved using acetate QuEChERS with liquid nitrogen for clean-up. For validation, organic olives were ground and spiked at 4 concentrations with pesticides and mycotoxins ($n = 5$). The linearity of the calibration curves was assessed by analyzing calibration standards of 7 concentrations which were prepared separately in acetonitrile and in blank olive extract ($n = 5$). The validation study demonstrated that the calculated r^2 was ≥0.99 for 144 pesticides and 6 mycotoxins, when the calibration curves were prepared in matrix extract, showing satisfactory linearity. Matrix effects were within the range of ±20% for only 46 pesticides and one mycotoxin. Then, to ensure reliable quantification, calibration standards had to be matrix-matched. In accuracy experiments 138 pesticides and 6 mycotoxins presented recoveries from 70 to 120% and RSD ≤ 20% for at least 2 of the 4 spike concentrations evaluated, being successfully validated. The integrated QuEChERS and LC-MS/MS method meet MRL for 11 of the 21 pesticides regulated for olives in Brazil and for 132 pesticides which are regulated in the EU law. Eleven commercial table olive samples were analyzed and 4 of them tested positive for pesticides. All the positive samples violate the Brazilian law and one sample violates also the European law.

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Introduction

Native to the Mediterranean, the olive trees are members of the Oleacea family. The gender *Olea* has several species, even though *Olea europaea* is the only one with commercial importance.[1] Because of its qualities, olives have been introduced in almost all continents.[2] Olives are rich in oil (12 to 30%) and are usually cured by fermentation processes to be consumed as pickled olives. The fruit can also be ground raw and later centrifuged (or pressed) for oil extraction. Along with grapes and wheat, olives are a member of the Mediterranean triad, the suite of plants underpinning ancient agriculture in the region.[3]

In Brazil, olive trees were introduced in the colonial period mainly in the southern states. Nevertheless, to avoid competition, the groves were cut down by order of the Portuguese crown. This fact prevented olive growing from taking the first impulse in Brazil.[4] This scenario only started to change recently. Currently, olive groves in Brazil cover about 7000 hectares, manly located in the state of Rio Grande do Sul (around 4500 hectares) and in a region denominated "Serra da Mantiqueira" (around 2000 hectares), which encompasses areas of the states of Minas Gerais, São Paulo and Rio de Janeiro.[5]

According to the statistics of the Food and Agriculture Organization of the United Nations (FAO), olive production in Brazil leapt from 77 tons in 2011 to 2651 tons in 2020 and to 3417 tons in 2021.[6] Contradictorily, Brazilian table olive consumption was around 124 000 tons in 2020,[7] which is 46.8 times higher than the production in the same year. Such data show that indeed domestic production is by far not enough to meet the consumption demand. In addition, Brazilian olive production is still very small when compared to other Latin American countries as Argentina, Peru and Chile, which produced 341 306; 147 011 and 130 344 tons respectively, in 2021.[6]

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As any other crop olives are subjected to pests, diseases and weeds during cultivation which can negatively affect production.[8] Mycotoxigenic fungi can contaminate olive fruits, and under favorable conditions, produce mycotoxins. These can also be transferred to table olives and olive oil.[9,10] Besides the fungi, insect pests are also of great relevance. Some pests as *e.g.* the olive fly, olive moth, psyllids, thrips, cochineal, neiroun and leopard moth can attack the groves damaging the fruits and cause decrease in production.[8]

Aiming to protect the fruits and maintain high productivity, pesticides are systematically applied to olive groves. The application of pesticides according to the good agricultural practices protects the plants and brigs benefits. However, the application of excessive amounts, use of banned pesticides, the non-compliance of preharvest interval or even the drift of pesticides applied to other crops can offer health risk to the consumers. To ensure food safety, many countries have set maximum residue levels (MRL) for pesticides in olives, and consequently, reliable analytical methods have become essential.[8]

According to SANTE N. 11312/2021,[11] olives belong to the group of commodities with high oil content and intermediate water content. The challenging task about multi-residue methods for such matrices is due the sample preparation approach. The high oil content must be removed from the extracts before chromatographic analysis, which requires efficient clean-up procedures, otherwise it can lead to unsatisfactory recoveries and/or high matrix effects.[12,13]

Over the last decades a considerable number multi-residue methods were reported for olive oil. Some of them covering small scopes (≤70),[14–18] others covering wide scopes (>100).[19–21] However, not so many methods were reported for pesticides determination in table olives[15,21,22] and even less were reported for mycotoxins. Since there is evidence that mycotoxins can contaminate olives[23–25] and olive oils,[26–33] it can be considered a concerning topic.

Many studies have also already addresses the contamination levels of olive oils with pesticides.[14,16,18,20,34] Several positive samples were reported by these articles and in some cases, the detected pesticides exceeded their MRL.[16,20,21,35] Among the determined pesticides were chlorpyrifos-ethyl,[20,21,34,35] diazinon,[20,21,34] thiacloprid,[14,21] quinalphos,[16,35] tetraconazole, dimethoate,[20,21] endosulfan-sulfate, fenthion[21,35] and parathion-methyl,[34,35] among others. Even the banned organochlorine insecticide 4,4-DDE was found at 15.7 μg kg^{-1} in one sample.[18]

Regarding olives contamination with pesticides, a study conducted by García-Vara *et al.* (2022)[15] analyzed samples from Iberian Peninsula produced from 2018 to 2020. A few samples tested positive for imidacloprid (>100 μg kg^{-1}), acetamiprid, desisopropyl-atrazine (DIA), terbutryn and irgarol.

Taghizadeh *et al.* (2021),[22] tested 1800 olive samples from Iran for the presence of 22 pesticides, besides other contaminants. Positive samples were reported and fortunately, all pesticides were in accordance with their corresponding MRL. Another study from Anagnostopoulos and Miliadis (2013)[21] evaluated olives an olive oils samples from Greece. Seven samples tested positive. The determined pesticides were chlorpyriphos-ethyl, dimethoate, omethoate and pyriproxyfen, some of them exceeded their MRL.

In addition to pesticides, mycotoxins contamination has also been detected in olive oils. Among the mycotoxins found are aflatoxin B1 at concentrations up to 2.4 μg kg^{-1},[31,32] alternariol (from 3.0 to 28.0 μg kg^{-1}),[26,27,30,33] alternariol monomethyl ether (up to 14.0 μg kg^{-1})[26,30] ochratoxin A (up to 17.7 μg kg^{-1}),[31,32] zearalenone (up to 7.1 μg kg^{-1}),[26,28,29] aflatoxins G1 (up to 1.9 μg kg^{-1}) and G2 (up to 6.8 μg kg^{-1}),[28] besides sterigmatocystin, tentoxin, beauvericin, mycophenolic acid, enniatin A, A_1, B and B_1 [26] and tenuazonic acid.[30]

In contrast, the subject of mycotoxins contamination in olives has not yet been explored so deeply, although a few studies are available in the literature. Khalil, Hashem and Abdelaziz (2019)[25] isolated mycotoxigenic *Penicillium* species from green table olives from Saudi Arabia. These authors used thin layer chromatography (TLC) for qualitative detection of citrinin, penicillic acid and cyclopiazonic acid. All samples stored at 10 to 15 °C for 21 to 30 days tested positive for mycotoxins produced by *Penicillium* species.

Franzetti *et al.* (2011)[24] analyzed 40 samples of green table olives from Italy. Aflatoxin B_1 was detected in 25% of the samples (in the range of 0.4 to 0.5 μg kg^{-1}), while ochratoxin A was found in 58% of the samples (in the range of 0.2 to 3.9 μg kg^{-1}).

El Adlouni *et al.* (2006)[23] investigated the presence of mycotoxins in black olives from Marocco. These authors reported the occurrence of ochratoxin A, citrinin and aflatoxin B in some samples. Seven samples tested positive for ochratoxin A at concentrations up to 0.6 μg kg^{-1}. Citrinin was determined in 5 samples (up to 0.5 μg kg^{-1}) and aflatoxin B was found in 4 samples at concentrations from 0.5 to 5 μg kg^{-1}.

Unfortunately, so far there are no maximum levels (ML) established in law for mycotoxins in olives or olive oils. Thus, this study aimed to optimize and validate an high performance QuEChERS based approach for pesticides and mycotoxins determination in the oily olive matrix using LC-MS/MS, and afterwards, to apply it to assess the contamination levels of olives samples commercialized in Brazil.

Experimental

Chemicals and reagents

Acetonitrile pesticide grade (99.9%), formic acid and acetic acid both analytical grade (99.8%) were purchased from J.T. Baker (Phillipsburg, NJ, USA). Ultrapure water was obtained from a Milli-Q Gradient Water System (Millipore, Milford, USA). Sodium acetate analytical grade was purchased from J.T. Baker (Xalostoc, Mexico). Anhydrous magnesium sulphate, sodium chloride, sodium citrate, sodium hydrogencitrate sesquihydrate and enhanced matrix removal-lipid (EMR-Lipid) SPE cartridges were supplied by Agilent (Folsom, CA, USA).

Pesticide standards (purity >97%) were obtained from Dr Ehrenstorfer (Augsburg, Germany). Mycotoxins standards were from Fermentek Biotechnology (Jerusalem, Israel) or from Sigma-Aldrich (Steinheim, Germany).

Standard solutions

Pesticides. Individual standard stock solutions of the 150 pesticides were prepared at the concentration of 1 mg mL^{-1} by weighting 10 mg of the reference material directly into a glass bottle and dissolving it in a specific volume of acetonitrile, considering the purity of the solid pesticide standard. Each solution was then taken to the ultrasonic bath to obtain a complete dissolution of the solid. A mixture solution of all pesticides was prepared in acetonitrile (at 1 μg mL^{-1}) by transferring calculated volumes of the standard stock solutions into a 500 mL volumetric flask and making the volume up to 500 mL with acetonitrile.

Mycotoxins. For the mycotoxins, the individual stock solutions of the 7 mycotoxins were prepared by dissolving the total amount of reference material contained in the original flask in order to avoid losses. Depending on their sensitivity into the LC-MS/MS system, the mycotoxins were classified into group 1 (G1) or group 2 (G2). G1 consisted of aflatoxins (B1, B2, G1 and G2) and ochratoxin A, G2 consisted of citrinin and zearalenone. For the aflatoxins, the stock solutions were prepared in methanol at 500 mg L^{-1} and for ochratoxin A in toluene/acetic acid (99 : 1). In case of G2 of mycotoxins, stock solutions were prepared in acetonitrile, at 500 mg L^{-1} for citrinin and at 1000 mg L^{-1} for zearalenone. Afterwards, a mixture standard solution of the 7 mycotoxins was prepared in acetonitrile by successive dilution of the individual stock solutions. Concentration of the mixture standard solution was of 1 μg mL^{-1} for mycotoxins of G1 and at 50 μg mL^{-1} for mycotoxins of G2. All standard solutions were stored in a freezer at −18 °C until use.

The mixture standard solutions of both mycotoxins and pesticides were used as spiking solutions to perform recovery experiments and to prepare calibration standards for a linearity study.

Internal standards. Two internal standards were used for quality control. Quinalphos was used as procedure internal standard (P.I.S.) and propoxur was used as instrument internal standard (I.I.S.). Both internal standards stock solutions were prepared in acetonitrile at 1 mg mL^{-1}. Working solutions were prepared by dilution of stock solutions. For quinalphos the working solution was of 2 μg mL^{-1} and for propoxur of 10 μg mL^{-1}.

The P.I.S. was spiked to the samples previously to extraction and used to ensure the correct execution of QuEChERS approach by calculating mean recoveries and repeatability relative standard deviation (RSD). Acceptance criteria were recoveries of 70 to 120% and RSD ≤ 20%.

The I.I.S. was added to the final dilution solvent (acetonitrile/water, 1 : 1) and used to ensure the accurate injection of sample extracts. RSD among the peak areas were calculated and injections were considered correctly done when RSD ≤ 20%. Internal standards were not used for correction or quantification purposes.

LC-MS/MS conditions

Chromatographic analysis was performed on an 1260 Infinity II Prime LC (Agilent, Waldbronn, Germany) coupled to an Ultivo triple quadrupole mass spectrometer (TQ-MS/MS) (Agilent, Singapore). The LC system was equipped with a flexible quaternary pump, multisampler and column oven.

Injection volume was 2 μL. Separation of the pesticides and mycotoxins was conducted on an InfinityLab Poroshell 120 SB C18 (100 × 3.0 mm i.d., 2.7 μm particle size) (Agilent, USA), kept at 45 °C. The column was connected to a guard column 3 PK InfinityLab Poroshell HPH-C18 (5 × 2.1 mm i.d., 2.7 μm particle size) (Agilent, USA).

The mobile phase consisted of ultrapure water containing 0.1% formic acid (eluent A) and acetonitrile containing 0.1% formic acid (eluent B). Gradient elution started with 80% eluent A (kept for 5 min), followed by a linear gradient down to 10% eluent A at 9 min (kept for 0.25 min) and then to 5% of eluent A at 11 min. Gradient was set gradually back to the initial condition at 13 minutes and the column was re-equilibrated for 1 minute, resulting in a total run time of 14 min. Mobile phase flow rate was set at 0.3 mL min^{-1} and it was diverted to waste during the first 0.5 min of the chromatographic run.

The mass spectrometer was equipped with an Agilent Jet Stream Source (AJS), which was operated in the positive and negative electrospray (ESI) mode. Nitrogen (N_2) was used as nebulizer gas (flow rate 11 L min^{-1} at 250 °C) and as sheath gas (flow rate 12 L min^{-1} at 350 °C). Capillary voltage and current were 3845 V and 5781 nA, respectively. The Ultivo TQ-MS/MS was operated in the dynamic multiple reaction monitoring mode (dMRM). Two transitions were monitored for each analyte. The optimized LC-MS/MS conditions for pesticides and mycotoxins are summarized in ESI Table S1.†

Data acquisition and processing was done by the MassHunter software, version 1.2 (Agilent, USA).

Samples and pretreatment

In order to provide blank sample (free of the studied pesticides and mycotoxins) for method optimization and validation, a sample of organic table olives (1 kg) was acquired from a certified organic producer located in the state of Minas Gerais, Brazil. This sample was previously analyzed to ensure it was really blank.

Aiming to assess the contamination levels of conventional table olives, 11 commercial samples of 0.5 kg and of different brands (with 3 replicates each, $n = 3$) were purchased in local markets of Santa Maria, Rio Grande do Sul, Brazil.

All olive samples had their pits removed and were then ground using a universal fruit multiprocessor (Philco, Brazil) until obtaining an homogeneous paste. Afterwards, the ground samples were stored in a freezer at −18 °C until analysis.

Slurried samples (diluted 2-fold) were used for method optimization (experiments II and IV, described in Fig. 1). The slurry was prepared directly into the 50 mL centrifuge tubes. For this, portions of 5 or 10 g of olive paste were weighed into the tubes, the P.I.S. was added and the tubes were homogenized in vortex for 30 s. For recovery experiments, spiking solutions were added and the tubes were homogenized in vortex again for 1 min. Finally, ultrapure water was added and the tubes were shaken in vortex for more 1 min.

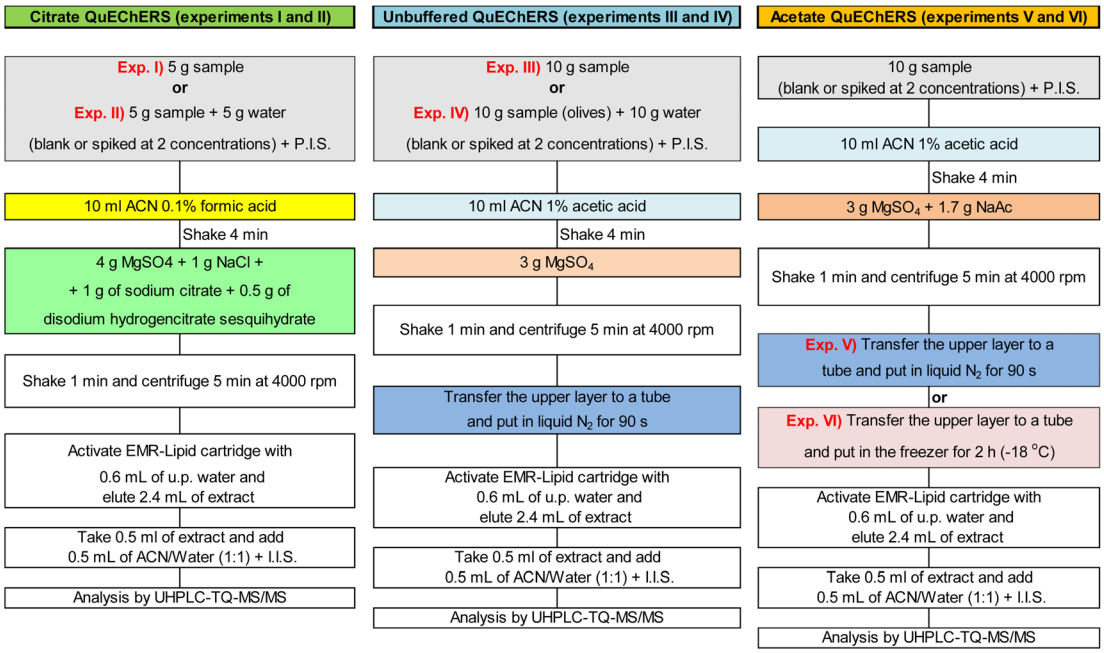

Fig. 1 Scheme of the QuEChERS approaches evaluated for olives.

Optimization and selection of the extraction method (QuEChERS approach)

Extraction experiments were conducted with 5 or 10 g of sample, with and without sample dilution (slurry) previously to extraction. Unbuffered, as well as citrate and acetate buffering versions of QuEChERS approach were evaluated in combination with clean-up steps of liquid nitrogen or 2 hours in freezer (−18 °C) for freezing out. Fig. 1 shows a scheme of QuEChERS approaches tested (experiments I, II, III, IV, V and VI).

Before extraction, the samples were spiked with pesticides at 10 and 50 μg kg^{-1} and with mycotoxins at 2 and 20 μg kg^{-1} for G1 and at 100 and 1000 μg kg^{-1} for G2 (with 3 replicates at each concentration, $n = 3$). Spiked samples were then extracted with QuEChERS approaches as demonstrated in Fig. 1.

Results obtained from those 6 experiments were compared and to select for validation one of the tested approaches the following parameters were considered: (i) ability to provide acceptable recoveries and RSD, besides low matrix effects; (ii) good cost-benefit relation; (iii) work and time required to perform the extraction approach.

Validation of the analytical method

According to SANTE N. 11312/2021,[11] laboratory method validation is done to assure that the method fits for the intended purpose. Therefore, this method validation study was conducted according to the mentioned guide criteria. Quantitative methods have to be assessed for sensitivity/linearity, matrix effects, limit of quantification (LOQ), specificity, recovery, precision and robustness.

In this study all 150 pesticides and 7 mycotoxins were extracted and determined simultaneously in one single chromatographic run.

Recovery experiments were performed to assess the accuracy (as trueness and precision) by spiking pesticides and mycotoxins to the blank olive matrix. The spike concentrations were 10, 20, 50 and 70 μg kg^{-1} for pesticides; 2, 10, 20 and 30 μg kg^{-1} for mycotoxins of G1 and 100, 500, 1000 and 1500 μg kg^{-1} for mycotoxins of G2. Five replicates ($n = 5$) were prepared at each spike concentration and the blank sample was also extracted 5 times ($n = 5$) to assess specificity.

The determination of the LOQ was done based on the lowest spiked concentration that meets criteria of recoveries within the range of 70–120% with an associated repeatability RSD ≤ 20%.[11]

Linearity of the calibration curves was assessed by analyzing calibration standards of 7 concentrations which were prepared separately in acetonitrile and in a blank olive extract (matrix-matched standards). Calibration standards were injected into the LC-MS/MS system 5 times each ($n = 5$) and the medium peak areas were considered for calculations. For pesticides, the concentrations of the standard solutions were: 1, 5, 10, 25, 35, 50 and 75 μg L^{-1} (corresponding to 2, 10, 20, 50, 70, 100 and 150 μg kg^{-1} of matrix). For mycotoxins of G1 concentrations were: 0.5, 1, 5, 10, 15, 25 and 50 μg L^{-1} (corresponding to 1, 2, 10, 20, 30, 50 and 100 μg kg^{-1}); for G2: 25, 50, 250, 500, 750, 1250 and 2500 μg L^{-1} (corresponding to 50, 100, 500, 1000, 1500, 2500 and 5000 μg kg^{-1}).

Matrix effects were calculated individually for each pesticide and mycotoxin based on the slope of calibration curves of each compound by using the equation below.

$$\text{Matrix effects (\%)} = [(a_1/a_2) - 1)] \times 100$$

a_1: slope of the calibration curve in matrix extract. a_2: slope of the calibration curve in acetonitrile.

Results and discussion

Method optimization and selection

Aiming to select for validation the most "fit for purpose" QuEChERS approach, samples spiked at two concentrations were analyzed and results are shown in Fig. 2.

Extraction of slurried samples was evaluated because in some cases, the addition of water to the samples (prior to extraction) can improve extraction efficiency. Sample dilution is generally recommended for low moisture commodities as cereals.[11] As demonstrated in Fig. 2(A and B), extraction of slurried samples (experiments II and IV) did not improve recoveries for pesticides or for mycotoxins.

Considering only the pesticides (Fig. 2A), the experiments that provided recoveries from 70 to 20% and RSD $\leq$ 20% for the largest number of pesticides were experiments I, II (for the highest spike concentration), III and V (for both spike concentrations). This last experiment yielded satisfactory recoveries for 83% of the pesticides at the spike concentration of 10 μg kg^{-1} (target LOQ), possibly because 10 g of sample was used for extraction rather than 5 g, as used in experiments I and II.

For the mycotoxins (Fig. 2B), experiments I, III, II, V and VI, respectively, presented the largest number of mycotoxins meeting satisfactory recovery and RSD criteria. Aflatoxins presented satisfactorily recoveries in all experiments at 20 μg kg^{-1}, except aflatoxin G2 in experiment IV, in which it was not detected. Citrinin presented recoveries (and RSD) of 89% (8%) and 92% (3%) for the spike concentration of 1000 μg kg^{-1} in experiments I and II, respectively. But it presented recoveries <70% for both spike concentrations in experiments III to VI. This may have been caused either by the pH of the extraction solvent or by the freezing out clean-up. Ochratoxin A was satisfactory recovered in experiments I to IV at the spike concentration of 20 μg kg^{-1}, while in experiments V and IV it presented recoveries <35%, probably due to the pH change caused by the acetate buffer. Zearalenone presented similar recovery and RSD results for all experiments, being apparently little affected by the different QuEChERS approaches tested.

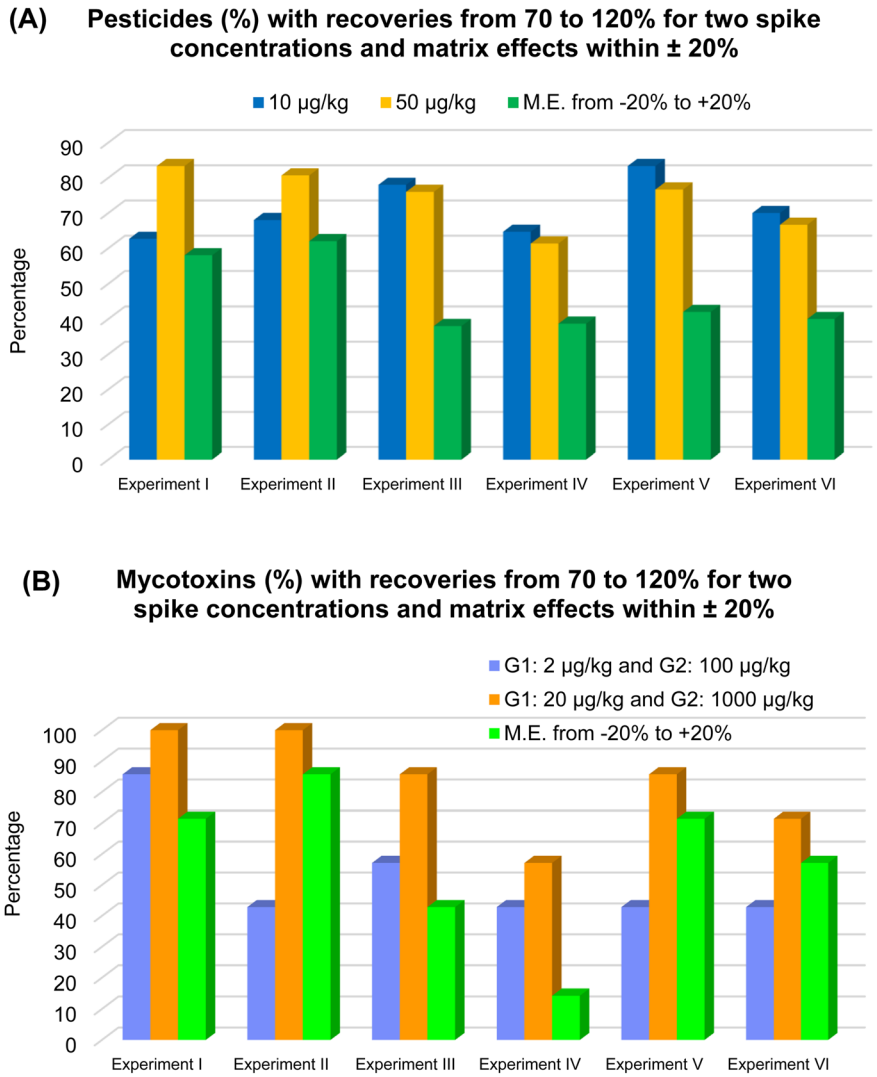

Fig. 2 Results of QuEChERS approach optimization study according to the six experiments (I, II, III, IV, V and VI) performed for pesticides (A) and for mycotoxins (B).

Regarding the matrix effects, experiments II, I and V, respectively, yielded matrix effects from −20 to +20% for more than 40% of the of pesticides and mycotoxins. In experiments I and II, the lower matrix effects can be explained by the smaller amount of sample used for extraction, 5 g instead of the 10 g. As less sample was extracted, less matrix interferers were available for co-extraction. In experiment V, the liquid nitrogen freezing out may have played a key role in reducing matrix effects for the mycotoxins. For pesticides, the liquid nitrogen clean-up was not able to overcome matrix effects, but it seems to have improved recoveries of some target pesticides, which is of utmost importance to achieve lower limits of quantification.

The worth results were obtained in experiment IV both for pesticides and mycotoxins. In this experiment, 10 g of sample was slurried with 10 g of water, and subsequently extracted with unbuffered QuEChERS. Apparently, the lack of the buffering step caused low recoveries (<70%) for many compounds, in special for target pesticides as buprofezin, diazinon, imidacloprid and pyrimethanil, besides the mycotoxins, aflatoxin G2 and citrinin. In addition, experiment IV was the one that presented the highest matrix effects.

In general, approaches of experiments I and V provided the best results of recovery and RSD and lower matrix effects. However for the pesticides, approach of experiment V yielded the best recovery results for the lowest spike concentration (10 μg kg^{-1}). Moreover, approach of experiment V (acetate QuEChERS) uses only two salts, one for buffering and one to removing water, while approach of experiment I (citrate QuEChERS) uses 2 salts for buffering and 2 to remove water, being more time, work and money costly per extracted sample. Therefore, acetate QuEChERS was chosen for validation.

Validation of the analytical method

Linearity. Aiming to assess the linearity, calibration standards of 7 concentrations were prepared in acetonitrile and in blank olive extract. Standards were injected 5 times each as described above. Data on the calibration curves are summarized in Table 1.

Determination coefficients and the deviations of back-calculated concentrations from true concentrations were calculated to estimate the fit of calibration curves into the linear function for all standards of the calibration curves. In case of the calculated deviation was out of range of ±20% for any specific calibration standard, this one was considered as not belonging to the calibration curve.[11] In this study, the calibration curves of all pesticides and mycotoxins had at least 5 calibration points (standards) for r^2 and the linear range estimation.

Empirically, calculated deviations outside the range of ± 20% are often observed at the lowest calibration points, which may then be removed from calibration curves for a better fit into the linear model.

As shown in Table 1, the calibration curves of more than 95.0% of the pesticides and 85.7% of the mycotoxins presented determination coefficients ≥0.990 that represents a proper fit to the linear function. A smaller percentage of the studied pesticides and mycotoxins presented determination coefficients in the range of 0.950 to 0.989, which is still considered acceptable for quantification purposes.

Matrix effects. Results of matrix effects are shown in Table 1 and in Fig. 3. From 150 pesticides and 7 mycotoxins, only 46 pesticides (30.7%) and one mycotoxin, namely, aflatoxin G1 (14.3%), presented matrix effects within the range of ±20%. Those other 104 pesticides (69.3%) and 6 mycotoxins (85.7%) presented matrix effects outside the mentioned range, which means that calibration curves have to be prepared in matrix extract to ensure reliable quantification. The majority of the compounds presented also medium (from −50% to −21%) or high (<−50%) negative matrix effects (ion suppression). In total, 138 pesticides (92.0%), and 6 (85.7%) mycotoxins presented

Table 1 Determination coefficients (r^2), linear ranges and matrix effects calculated from the calibration curves for 150 pesticides and 7 mycotoxins

		Number of pesticides (% of pesticides)		Number of mycotoxins (% of mycotoxins)	
		Standards in acetonitrile	Matrix-matched standards	Standards in acetonitrile	Matrix-matched standards
r^2	≥0.990	144 (96.0%)	143 (95.3%)	6 (85.7%)	6 (85.7%)
	From 0.980 to 0.989	4 (2.7%)	6 (4.0%)	0 (0.0%)	0 (0.0%)
	From 0.950 to 0.979	2 (1.3%)	1 (0.7%)	1 (14.3%)	1 (14.3%)
Linear range (μg L^{-1})	Pesticides: from 1 to 75 mycotoxins G1: from 1 to 50	34 (22.5%)	30 (19.9%)	4 (57.1%)	3 (42.8%)
	Pesticides: from 5 to 75 mycotoxins G1: from 5 to 50	112 (74.8%)	111 (74.2%)	1 (14.3%)	2 (28.6%)
	Pesticides: from 10 to 75 mycotoxins G2: from 50 to 2500	4 (2.7%)	9 (5.9%)	1 (14.3%)	1 (14.3%)
	Pesticides: from 25 to 75 mycotoxins G2: from 250 to 2500	0 (0.0%)	0 (0.0%)	1 (14.3%)	1 (14.3%)
Matrix effects	Within the range of ±20%	46 (30.7%)		1 (14.3%)	
	Outside the range of ±20%	104 (69.3%)		6 (85.7%)	

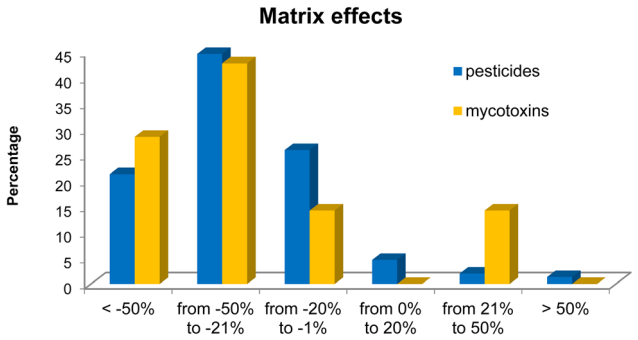

Fig. 3 Results of matrix effects for the studied pesticides and mycotoxins.

negative matrix effects when compared to only 12 (8.0%) pesticides and one (14.3%) mycotoxin (ochratoxin A) that presented positive matrix effects (signal enhancement). Negative matrix effects were already expected because of the ionization suppression caused by the co-eluted matrix compounds in the Jet Stream ESI source.[36,37]

Recovery, precision and LOQ. Recovery experiments were performed by spiking blank olive samples at 4 concentrations (n = 5). Results are shown in Tables 2 and 3 for pesticides and mycotoxins, respectively.

Considering Table 2, it is possible to observe that from a total of 150 pesticides, 138 (92%) fulfilled the validation requirements and were consequently successfully validated. While 10 pesticides (6.7%) did not fulfil validation requirements due to recoveries outside the range of 70–120% and/or RSD > 20% and 2 (1.3%) were not detected at any of the spike concentrations studied.

As summarized in Table 3, 6 (85.7%) mycotoxins out of 7 met validation requirements. Only one, ochratoxin A (14.3%), was not validated due to too low recoveries, despite it was still detectable at concentrations ≥10 μg kg^{-1}.

The low recoveries (<40%) obtained for ochratoxin A may be explained by two factors: the pH of the extraction solution and the clean-up step. The authors know already from previous studies[38] that some mycotoxins are affected by the pH of the extraction solvent and can also be retained in clean-up adsorbents, resulting in low recoveries.

As described before, limits of quantification were stated according to SANTE[11] criteria, which considers the LOQ the lowest concentration of the analyte that has been validated with acceptable accuracy. For pesticides (Table 2) the LOQ was of 10 μg kg^{-1}, 20 μg kg^{-1} and 50 μg kg^{-1} for 130 (86.7%); 3 (2.0%) and 5 (3.3%) of the pesticides, respectively. The other 12 pesticides (8%) did not fulfil validation requirements or were not detected at any spike concentration studied.

In Brazil, a total of 21 pesticides are allowed by law for the olive culture. The MRL are in the range of 0.01 to 5 mg kg^{-1} depending on the pesticide.[39] From the 21 regulated pesticides, 12 were covered by the method of this study and 11 were successfully validated presenting LOQ ≤ MRL for olives (Tables 2 and ESI S2†). The method is suitable for assessing compliance of these 11 pesticides with the MRL in olives and the other 127 validated pesticides can contaminate olives either by illegal use or by drift, when they are applied to other cultures grown close to olives groves, playing an important key role in the method.

Thiophanate-methyl, which is regulated in Brazil and covered by the method, did not meet validation requirements because it was not detected in recovery experiments. Thiophanate-methyl is a tricky pesticide. It belongs to the group of the benzimidazol fungicides and may suffer degradation into carbendazim.[40]

Within the EU the number of compounds (pesticides, growth regulators, metabolites, *etc.*) allowed for olive plantation is over 500, with MRL ranging from 0.003 to 20 mg kg^{-1}.[41] The method of this study covers 132 pesticides (ESI Table S2†) which are regulated in the EU law and, from these, 114 pesticides have a suitable LOQ (LOQ ≤ MRL) to assess compliance to the European MRL for olives. Just eight pesticides presented LOQ > MRL. However, they are still detectable.

In addition, from those 138 pesticides which were successfully validated in this study, 15 are not approved for use in the EU and 1 (piperonyl butoxide) was not yet assessed at the EU level. These pesticides can contaminate olives by illegal use and/or drift.

Table 3 summarizes LOQ for mycotoxins. The LOQ was stated at 2 μg kg^{-1} for 3 (42.8%) and at 10 μg kg^{-1} for 1 (14.3%) mycotoxin of G1. Both mycotoxins of G2 (28.6%) had LOQs of 500 μg kg^{-1}. Neither the Brazilian legislation nor the EU legislation establishes maximum levels (ML) for mycotoxins in table olives.[42,43]

Quality control using internal standards

Quinalphos was used as the P.I.S. and it was spiked to the samples previously to extraction. A P.I.S. is added to the analytical test portion prior to the extraction step to account for various sources of errors throughout all stages in the method.[11] In the present study, recoveries and RSD were calculated for quinalphos to monitor possible errors. As shown in Table 2, quinalphos presented recoveries in the range of 77 to 99% with associated RSD from 1 to 5%, which meets the acceptance criteria and demonstrates the correct execution of the method.

Propoxur was used as the I.I.S. and was added to the final dilution solvent. RSD among propoxur's peak areas were calculated. According to SANTE,[11] an I.I.S. is added to the final extracts, just prior to injection to allow a check and possible correction for variations in the injection volume. Propoxur presented an RSD from 2 to 8% for the 4 spike concentrations, representing an acceptable variation in the samples injection volume.

Sample analysis

Some commercial table olive samples were analyzed with the validated method aiming to assess compliance with the MRL. From 11 samples, 4 tested positive for pesticides.

Buprofezin was found in 2 samples at 0.010 mg kg^{-1} in the first sample, and at 0.033 mg kg^{-1} in the second one. This pesticide is not allowed for the crop in Brazil, consequently both samples violate the Brazilian law. In EU buprofezin is allowed

Table 2 Results of recovery (%), RSD (%) and limit of quantification for pesticides spiked to blank olives at four concentrations[ab]

	Spike concentrations								
	10 μg kg^{-1}		20 μg kg^{-1}		50 μg kg^{-1}		70 μg kg^{-1}		LOQ
Pesticide	Rec. (%)	RSD (%)	Rec. (%)	RSD (%)	Rec. (%)	RSD (%)	Rec. (%)	RSD (%)	(μg kg^{-1})
Acephate	109	5	101	6	89	6	89	16	10
Acetamiprid	112	5	98	3	97	3	98	3	10
Acetochlor	99	7	77	7	80	11	98	7	10
Aldicarbe sulfone	117	4	105	5	97	2	80	8	10
Aldicarbe sulfoxide	n.d.	n.d.	72	45	51	15	44	13	n.f.r.
Atrazine	107	3	96	2	94	2	80	9	10
Azamethiphos	118	6	101	5	99	7	81	9	10
Azinphos-methyl	87	9	103	13	107	5	93	12	10
Azoxystrobin	119	4	109	2	111	4	90	9	10
Benfuracarb	170	87	90	66	121	76	85	77	n.f.r.
Bifenazate	97	8	91	9	95	8	75	14	10
Bitertanol	107	12	92	8	95	10	79	12	10
Boscalid	92	11	94	3	98	6	78	15	10
Bromuconazol	102	10	90	3	92	3	81	13	10
Bupirimate	104	4	97	4	98	3	81	12	10
Buprofezin	97	3	83	3	81	8	80	3	10
Cadusaphos	97	3	87	11	97	18	94	15	10
Carbaryl	106	9	98	8	97	8	99	8	10
Carbendazim	n.d.	n.d.	n.d.	n.d.	13	70	13	49	n.f.r.
Carbofuran	114	9	118	5	115	9	117	4	10
Carpropamid	104	15	88	8	91	9	95	10	10
Chlorfenvinphos	107	14	96	12	104	12	104	13	10
Chlorpyrifos	87	47	72	19	85	15	70	9	20
Clofentezine	84	63	74	38	75	19	84	17	50
Clothianidin	118	10	105	3	105	3	107	5	10
Cyazofamid	116	11	98	15	119	6	118	9	10
Cyproconazole	82	11	93	7	94	5	98	3	10
Cyprodinil	73	9	72	5	71	4	74	1	10
Demeton-S-methylsulfone	115	6	100	3	100	3	101	4	10
Diazinon	93	9	91	4	93	7	95	7	10
Dichlofluanid	n.d.	n.d.	n.d.	n.d.	105	17	99	17	50
Diethofencarb	101	8	101	6	104	6	101	4	10
Difenconazole	101	3	86	4	83	7	84	3	10
Diphenylamine	98	13	89	7	78	6	77	7	10
Diflubenzuron	n.d.	n.d.	47	71	79	17	72	11	50
Dimethoate	114	3	98	2	97	2	100	2	10
Dimethomorph	108	4	101	4	102	5	101	4	10
Diniconazole	98	14	86	11	88	13	89	12	10
Diuron	102	13	101	6	101	5	102	2	10
Epoxiconazole	104	6	104	5	101	5	99	3	10
Ethion	87	13	80	9	84	8	81	3	10
Ethirimol	81	4	71	5	57	3	55	5	10
Etofenprox	110	13	92	16	72	8	70	6	10
Ethoprophos	90	8	94	7	96	8	90	7	10
Ethiprole	104	12	113	7	119	6	119	5	10
Etoxazole	87	3	81	3	79	3	78	3	10
Fenamidone	116	6	103	4	103	5	103	5	10
Fenamiphos	98	10	104	5	109	9	102	8	10
Fenarimol	95	8	90	7	82	11	85	7	10
Fenazaquin	68	2	60	3	57	6	57	4	10
Fenbuconazole	91	18	97	12	94	9	93	9	10
Fenhexamid	110	16	91	10	92	10	92	7	10
Fenobucarb	105	4	102	7	104	2	103	4	10
Fenoxycarb	94	15	95	6	97	9	104	11	10
Fenpropimorph	70	2	63	3	63	3	63	2	10
Fenpyroximate	81	4	72	3	70	3	71	3	10
Fensulfothion	103	7	120	7	119	4	114	1	10
Fluazifop-butyl	107	4	94	4	93	5	94	4	10
Fludioxonil	227	5	206	4	199	4	190	4	n.f.r.

Table 2 (*Contd.*)

	Spike concentrations								
	10 μg kg^{-1}		20 μg kg^{-1}		50 μg kg^{-1}		70 μg kg^{-1}		LOQ
Pesticide	Rec. (%)	RSD (%)	Rec. (%)	RSD (%)	Rec. (%)	RSD (%)	Rec. (%)	RSD (%)	(μg kg^{-1})
Flufenoxuron	93	15	96	17	93	10	93	8	10
Fluquiconazole	97	14	95	9	94	9	90	9	10
Flusilazole	92	11	94	3	98	6	94	7	10
Flutolanil	109	13	103	8	105	9	100	8	10
Flutriafol	118	7	113	11	108	9	108	8	10
Fostiazato	119	4	105	4	102	4	103	5	10
Furalaxyl	115	7	108	5	108	4	107	4	10
Furathiocarb	90	7	90	4	94	10	90	6	10
Halofenozide	105	12	107	14	114	7	109	10	10
Haloxyfop-2-ethoxyethyl	95	11	95	6	96	9	97	7	10
Hexaconazole	88	7	84	9	86	10	85	8	10
Hexythiazox	94	12	76	10	70	3	72	8	10
Imazalil	82	2	76	3	79	1	79	2	10
Imazapic	15	3	11	5	11	2	12	4	n.f.r.
Imidacloprid	120	3	105	1	102	2	103	3	10
Indoxacarb	116	14	103	18	94	19	94	19	10
Iprovalicarb	108	7	103	2	107	6	102	4	10
Kresoxim-methyl	119	10	110	10	111	16	105	11	10
Linuron	83	16	90	17	109	12	101	10	10
Malathion	119	6	111	10	112	6	115	7	10
Mecarbam	107	12	105	14	108	17	102	14	10
Mepanipyrim	71	11	76	9	79	9	102	14	10
Metalaxil	117	2	102	2	100	1	100	3	10
Metconazole	101	5	87	5	84	5	86	3	10
Methamidophos	88	6	79	3	79	3	77	3	10
Methidathion	90	16	116	12	117	6	116	8	10
Methiocarb	103	9	98	4	100	4	101	4	10
Methiocarb sulfone	95	13	87	5	82	4	85	5	10
Methiocarb sulfoxide	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Methomyl	108	3	95	3	95	3	96	3	10
Methoxyfenozide	309	8	270	6	255	6	241	7	n.f.r.
Monocrotophos	105	15	86	8	81	6	81	5	10
Myclobutanil	108	12	106	14	108	10	104	8	10
Nitenpyram	83	16	92	14	72	19	76	11	10
Ofurace	115	5	105	3	104	3	106	4	10
Omethoate	100	8	84	6	76	6	77	6	10
Oxadixyl	107	5	90	4	85	3	85	4	10
Oxamyl	120	3	105	4	101	3	101	3	10
Paclobutrazol	97	7	102	5	107	5	104	3	10
Penconazole	89	15	88	10	93	9	93	7	10
Pencycuron	90	9	84	9	91	11	91	10	10
Pendimethalin	88	13	73	12	71	8	71	4	10
Phenothrin	100	8	70	9	67	2	65	5	10
Phenthoate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Phosalon	n.d.	n.d.	114	19	96	19	106	19	20
Phosmet	71	16	105	13	116	7	111	7	10
Picoxystrobin	112	6	105	6	106	6	108	5	10
Piperonyl butoxide	95	4	87	5	89	3	87	5	10
Pirazophos	94	20	90	7	101	16	98	17	10
Pirimicarb	10	3	4	18	1	1	2	2	n.f.r.
Pirimiphos-methyl	85	10	85	6	88	9	87	7	10
Pirimiphos-ethyl	93	6	80	5	80	7	78	6	10
Prochloraz	81	4	88	3	91	4	94	3	10
Profenofos	98	9	74	10	75	12	74	10	10
Prometryn	88	2	78	2	75	1	75	3	10
Propamocarb	35	5	35	3	37	2	37	3	n.f.r.
Propanil	n.d.	n.d.	n.d.	n.d.	94	14	94	17	50
Propargite	92	9	83	8	79	6	81	7	10
Propham	105	13	99	8	98	6	100	6	10

Table 2 (*Contd.*)

	Spike concentrations								
	10 μg kg^{-1}		20 μg kg^{-1}		50 μg kg^{-1}		70 μg kg^{-1}		LOQ
Pesticide	Rec. (%)	RSD (%)	Rec. (%)	RSD (%)	Rec. (%)	RSD (%)	Rec. (%)	RSD (%)	(μg kg^{-1})
Propiconazole	89	13	88	11	91	12	94	8	10
Propyzamide	107	12	92	7	85	11	88	6	10
Propoxur (I.I.S.)	—	2	—	3	—	8	—	5	—
Pyraclostrobin	98	5	89	4	89	7	92	7	10
Pyridaben	118	4	91	4	77	8	74	6	10
Pyrimethanil	90	4	83	2	80	2	80	3	10
Pyriproxyfen	101	3	80	3	71	3	71	4	10
Quinalphos (P.I.S.)	99	5	84	4	77	1	77	2	—
Quinoxyfen	112	15	86	18	78	11	82	8	10
Simazine	89	3	78	3	73	3	75	2	10
Spinosyn D	51	6	50	10	58	3	64	5	10
Spinosyn A	56	4	54	8	61	8	67	2	10
Spirodiclofen	84	9	82	7	79	10	78	8	10
Spiromesifen	98	11	87	9	86	12	85	10	10
Spiroxamine	73	2	70	3	73	2	75	1	10
Tau-fluvalinate	n.d.	n.d.	101	16	87	13	93	10	20
Tebuconazole	75	8	90	8	92	7	90	9	10
Tebufenoxide	117	10	109	11	118	13	111	9	10
Tebufenpyrad	73	7	72	15	71	7	75	9	10
Tetrachlorvinphos	113	9	97	10	100	8	98	9	10
Tetraconazole	111	4	101	6	102	4	101	7	10
Tetramethrin	85	14	82	7	83	8	82	6	10
Thiabendazole	92	7	73	9	72	12	70	12	10
Thiacloprid	117	6	107	2	100	3	102	3	10
Thiamethoxam	72	110	53	49	54	29	49	19	n.f.r.
Thiodicarb	112	4	99	3	97	3	97	3	10
Thiophanate-methyl	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Triadimefon	110	5	93	8	91	6	86	7	10
Triadimenol	74	107	64	43	99	15	92	6	50
Triazophos	116	10	102	8	103	11	100	11	10
Trifloxystrobin	91	19	89	10	94	10	91	7	10
Triflumizole	92	4	87	4	89	6	90	5	10
Triticonazole	90	14	89	7	92	3	94	3	10
Zoxamide	85	8	89	19	87	19	92	17	10

[a] n.f.r.: Not fulfilling requirements for quantitative method (recovery: 70–120% and RSD ≤ 20%); n.d.: not detected. [b] I.I.S.: instrument internal standard; P.I.S.: procedure internal standard.

Table 3 Results of recovery (%), RSD (%) and limit of quantification for mycotoxins of groups 1 and 2 spiked to blank olives at four concentrations[a]

		Spike concentrations								
		G1: 2 μg kg^{-1}		G1: 10 μg kg^{-1}		G1: 20 μg kg^{-1}		G1: 30 μg kg^{-1}		
		G2: 100 μg kg^{-1}		G2: 500 μg kg^{-1}		G2: 1000 μg kg^{-1}		G2: 1500 μg kg^{-1}		
Mycotoxin	Group	Rec. (%)	RSD (%)	Rec. (%)	RSD (%)	Rec. (%)	RSD (%)	Rec. (%)	RSD (%)	LOQ (μg kg^{-1})
Aflatoxin B1	1	119	7	105	7	95	3	95	9	2
Aflatoxin B2	1	n.d.	n.d.	113	6	98	3	95	4	10
Aflatoxin G1	1	106	16	117	8	95	5	91	8	2
Aflatoxin G2	1	111	12	104	4	92	5	93	3	2
Citrinin	2	n.d.	n.d.	76	4	93	1	93	3	500
Ochratoxin A	1	32	63	36	11	35	12	34	7	n.f.r.
Zearalenone	2	n.d.	n.d.	99	13	97	14	98	10	500

[a] n.f.r.: Not fulfilling requirements for quantitative method (recoveries from 70–120% and RSD ≤ 20%); n.d.: not detected.

for olives up to the MRL of 0.01 mg kg^{-1}. Thereby, the last sample violates also the EU law.

Another 2 samples tested positive for buprofezin and pyrimethanil. The concentration of buprofezin was below the LOQ in the samples. Pyrimethanil was found at 0.014 mg kg^{-1} in one sample and below the LOQ in the other one. Both samples violate the Brazilian law, although they are in accordance with the MRL of the EU (ESI Table S2†). The chromatograms of some positive samples are shown in ESI Fig. S1.†

These results reinforce the need of monitoring pesticide residues in processed commodities in order to ensure food safety and compliance with the good agricultural practices. Moreover, concerning the olive cultivation demand in Brazil, a revision and update of the Brazilian legislation seems to be necessary to attend pests control actions in olive groves.

Regarding the studied mycotoxins, even with reports in literature of mycotoxins occurrence in table olives,[23–25] none of the samples analyzed in this study tested positive for mycotoxins.

Conclusions

Results of the method optimization experiments demonstrated that the three versions of QuEChERS approach were able to provide satisfactorily recoveries for more than 60% of the pesticides and mycotoxins studied. In general, citrate and unbuffered QuEChERS yielded better recoveries for mycotoxins while acetate QuEChERS provided better results for pesticides, especially at the target LOQ level of 10 μg kg^{-1}. Extraction of 5 g of sample, instead of 10 g, was advantageous in terms of lower matrix effects. However, by extracting 10 g of sample it was possible to achieve lower limits of quantification. The liquid nitrogen freezing out clean-up apparently played a key role in reducing matrix effects for mycotoxins and improved recoveries of some target pesticides. Based on these results, the approach of experiment V (acetate QuEChERS with liquid nitrogen for clean-up) was chosen for validation.

In the validation study, the analytical method presented satisfactory accuracy and precision for the majority of the studied compounds. From 150 pesticides, 92% were successfully validated. Concerning the 7 mycotoxins, 85.7% met validation requirements.

Finally, by the analysis of 11 commercial table olive samples it was possible to demonstrate the contamination of 4 of them with pesticide residues. Buprofezin was detected in all positive samples and pyrimethanil was detected in 2 of them. All the positive samples violate the Brazilian law and one sample also violates the EU law. Such results demonstrate the importance of food safety actions and the need of stricter enforcement of pesticide residues in table olives. Considering that the olive cultivation is growing a lot in Brazil, a revision of the policy for pesticides and its concentrations (MRL) allowed for this commodity seems to be necessary to meet cultivation's challenges.

Author contributions

Bárbara Reichert: methodology, investigation, validation, data processing, sample analysis, writing – original draft. Ionara Regina Pizzutti: supervision, writing – review and editing. Bárbara Daiana Jänisch: methodology, investigation and validation. Marlos Eduardo Zorzella Fontana: methodology, investigation, validation and data processing.

Conflicts of interest

There are no conflicts to declare.

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