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Abstract

The use of yttria-stabilized zirconium dioxide nanoparticles as d-SPE clean-up sorbent for a rapid and sensitive liquid chromatography–electrospray ionization-tandem mass spectrometry (LC–ESI-MS/MS) method for the determination of post-harvest fungicides (carbaryl, carbendazim, chlorpropham, diphenylamine, ethoxyquin, flutriafol, imazalil, iprodione, methomyl, myclobutanil, pirimiphos-methyl, prochloraz, pyrimethanil, thiabendazole, thiophanate-methyl and tolclofos-methyl) in orange and pear samples has been evaluated and validated. The sample preparation was a modification of the QuEChERS extraction method using yttria-stabilized zirconium dioxide and multi-walled carbon nanotubes (MWCNTs) nanoparticles as the solid phase extraction (d-SPE) clean-up sorbents prior to injecting the ten-fold diluted extracts into the LC system. By using the yttria-stabilized zirconium dioxide extraction method, more recoveries in the 70–120 % range were obtained – thus this method was used for the validation. Quantification was carried out using a matrix-matched calibration curve which was linear in the 1–500 μg kg−1 range for almost all the pesticides studied. The validated limit of quantification was 10 μg kg−1 for most of the studied compounds, except chlorpropham, ethoxyquin and thiophanate-methyl. Pesticide recoveries at the 10 and 100 μg kg−1 concentration levels were satisfactory, with values between 77 % and 120 % and relative standard deviations (RSD) lower than 10 % (n=5). The developed method was applied for the determination of selected fungicides in 20 real orange and pear samples. Four different pesticide residues were detected in 10 of these commodities; 20 % of the samples contained pesticide residues at a quantifiable level (equal to or above the LOQs) for at least one pesticide residue. The most frequently-detected pesticide residues were: carbendazim, thiabendazole and imazalil-all were below the MRL. The highest concentration found was imazalil at 1175 μg kg−1 in a pear sample.

Keywords: Post-harvest pesticides; Yttria-stabilized zirconium dioxide; Clean-up; LC–MS/MS; Pesticide residues; Recoveries

1. Introduction

Post-harvest treatment with fungicides is a common practice to prevent decomposition and diseases primarily affecting citrus fruits during storage or long-distance transport caused by pathogens such as green and blue moulds [1] and [2]. Among the most widely used post-harvest fungicides are carbendazim, imazalil, iprodione and the antioxidant diphenylamine [2], [3] and [4]. These compounds act as mitosis inhibitors, altering membrane function and inhibiting spore germination [5]. Nowadays, regulations have been established concerning Maximum Residue Levels (MRLs) for these post-harvest pesticides. For instance, under European Union (EU) regulation (EC) No 396/2005, the MRL for carbendazim on citrus fruits (such as oranges) is 0.2 mg kg^{-1}; for imazalil, it is 5 mg kg^{-1} and for iprodione, 0.02 mg kg^{-1} [6] and [7]. The European Food Safety Authority's (EFSA) annual report for 2011 [8] showed that the most frequently detected pesticides in 1461 oranges samples analysed were imazalil (detected in 64.5 % of the tested samples), chlorpyrifos (42.1 %) and thiabendazole (25.9 %). Furthermore, the most frequent MRL exceedances (in %) were recorded for imazalil, carbaryl and dimethoate. The same study was also carried out on pear samples showing that the most frequently detected pesticides in the 1364 pear samples analysed were dithiocarbamates (42.4 %), boscalid (23.7 %) and pyraclostrobin (16.8 %). The most frequent MRL exceedances (in %) were recorded for chlormequat, imazalil and carbendazim. In 2012, EFSA reported on the analysis of 695 orange juice samples showing that the most commonly detected pesticides were carbendazim (detected in 20.5 % of the tested samples) and imazalil (15.1 %) whilst no MRL exceedances were reported for these samples [9]. Therefore, monitoring fungicide residues in fruits is important to ensure food safety. Currently, one of the common pesticide sample preparation techniques is the

QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) extraction method. This is used as a reference to get high recovery from a broad scope of pesticides with different polarity and volatility in different matrices-low detection limits are obtained using smaller volumes of organic solvents and non-sophisticated equipments [10]. The method has been regularly used for all types of samples such as fat, oil, fruit, vegetables, rice, and bread [11] and [12]. For this reason, the method and its modified versions are extensively employed in food analysis and serve as a template for the determination of pesticide residues [13] and [14]. The complexity of certain matrices can cause problems with the ion production efficiency and with the analytical instruments' detection systems [15]. Consequently, there is interest in the introduction of any effective new extraction modification for matrix-dependent clean-up combinations. QuEChERS clean-up involves dispersive-solid phase extraction (d-SPE) which attempts to absorb interfering substances in the matrices rather than the analytes using anhydrous magnesium sulphate as the drying agent and primary secondary amine as the d-SPE sorbent (PSA); this removes polar organic acids, polar pigments, sugar, and fatty acids. It can work in combination with C18 (used to remove non-polar interference substances such as lipids) [16] and [17] and graphitized carbon black (GCB, used to remove pigments such as chlorophyll and steroids) sorbents for further clean-up [17] and [18]. The main disadvantage of PSA as the d-PSE sorbent is that some pH-sensitive pesticides (base-labile compounds) can suffer hydrolysis under basic conditions in the dispersive SPE resulting in pH values which may exceed 8 – thus making it necessary to use formic acid after clean-up to reduce pH-an undesirable additional step [19], [20] and [21]. Zhao et al., on the other hand, used multi-walled carbon nanotubes (MWCNTs) as an alternative reversed-dispersive solid phase extraction (r-DSPE) sorbent material to PSA/GCB/C18 for the clean-up of extracts in various matrices such as cabbage, spinach, grape and orange showing good results [22]. Zhao et al. also developed a clean-up applying this streamlined procedure to a multi-plug filtration clean-up column coupled to a syringe [23]. Although the sample clean-up process functioned without any solvent evaporation, vortex, or centrifugation procedure, the recoveries for benzo-heterocyclic compounds using multi-plug filtration clean-up (m-PFC) were not satisfactory. Magnetic nanoparticles such as Fe_3O_4 have been widely-used because of their particular magnetic, adsorption, optical, and mechanical properties as well as their extremely large specific surface area for application in the fields of analytical chemistry [24]. Although the experimental procedure benefits from easier operation steps and the recovery rate was higher, meeting the requirements for pesticides analysis, the preparation of Fe_3O_4 magnetic nanoparticles by chemical co-precipitation involves an additional step [25] and [26]. Consequently, a possible alternative to these clean-up combinations is to use yttria-stabilized zirconium dioxide nanoparticles (ZrO_2/Y_2O_3), which have a high surface area per m^2 (surface area >100 m^2/g), in the clean-up system to simultaneously obtain excellent purification effects and satisfactory results for multiple pesticides. As previously reported, sorbents containing ZrO_2, such as Z-Sep and Z-Sep+ (supplied by Supelco, Bellefonte,PA), can be used as the clean-up material for pesticide analysis in high oil matrices, such as avocado and almond; these are better than PSA or C18 at removing fatty acids, esters of fatty acids, sterols and carboxylic acids [27].

This work is focused on the evaluation of yttria-stabilized zirconium dioxide and multi-walled carbon nanotubes (MWCNTs) nanoparticles as the sorbents in the clean-up material for post-harvest pesticide analysis-thus modifying QuEChERS methodology, optimizing the experimental procedure and reducing costs. This modified QuEChERS method using zirconium dioxide nanoparticles in combination with liquid chromatography–tandem mass spectrometry (LC–MS/MS) was validated in orange and pear matrices.

2. Experimental

2.1 Chemicals and reagents

All high purity pesticide standards were obtained from Sigma-Aldrich (Steinheim, Germany) and Dr. Ehrenstorfer (Augsburg, Germany) and were stored at −30 °C. Isotope-labelled internal standards of Carbendazim-d_3, Dimethoate-d_6 and Malathion-d_{10} were purchased from Dr. Ehrenstorfer (Augsburg, Germany) and from CDN Isotopes (Quebec, Canada). Individual pesticide stock solutions (1000–2000 mg L^{-1}) were prepared in acetonitrile and were stored in amber screw-capped glass vials in the dark at −20 °C. Individual standard solutions for optimization and the standard-mix solution for calibration were prepared from the stock standards. Ultra-gradient HPLC grade acetonitrile was obtained from Sigma-Aldrich (Steinheim, Germany). Ultra-gradient HPLC-grade water was obtained from Thermo Fisher (Waltham, MA USA). Formic acid and trisodium citrate dihydrate were purchased from Fluka (Steinheim, Germany). Primary-secondary amine (PSA) Bond-Elut was obtained from Supelco (Bellefonte, PA, USA). Sodium chloride was purchased from J.T Baker (Deventer, Netherlands). Disodium hydrogen citrate sesquihydrate was obtained from Sigma-Aldrich (Steinheim, Germany). Anhydrous magnesium sulphate was supplied by Panreac (Barcelona,Spain). Yttria-stabilized zirconium dioxide, multi-walled carbon nanotubes (MWCNTs) of 6−9 nm×5 μm, zirconium dioxide nanopowder (surface area ≥25 m^2/g) were supplied by Sigma-Aldrich (Steinheim, Germany). Z-Sep (zirconia-coated silica) and graphitized carbon black (GCB) were supplied by Supelco (Bellefonte,PA).

2.2. Sample treatment for recovery studies

For the recovery studies, orange, and pear (blanks) obtained from a local market were spiked with the

standard solution of pesticides in acetonitrile at the appropriate levels before the corresponding extraction procedure. Samples had to be fortified with the mix by thoroughly soaking the entire sample and then stirring it to ensure effective homogenization. Following this, representative portions of the previously homogenized matrices were divided into appropriate amounts and weighed. The final spiking concentration levels in the samples for recovery studies were 10 and 100 μg kg^{-1}. In the case of ethoxyquin in pear matrix, to obtain satisfactory recoveries within the 70−120 % range, the matrix had to be spiked with the mix as described above, acidified with formic acid at pH 3 and then homogenized in an automatic axial extractor for 15 min.

2.3. Extraction by QuEChERS and the modified methods

For method validation, we used the QuEChERS method on the different matrices following the protocol previously reported [28]. For the extraction, 10 g of sample was used. The orange and pear samples were mixed with 10 mL of acetonitrile by automatic shaking in an automatic axial extractor (AGYTAX®, Cirta Lab. S.L., Spain) at ambient temperature for 4 min. This mixture was placed in a centrifuge tube containing 4 g of anhydrous magnesium sulphate, 1 g of sodium chloride, 1 g of trisodium citrate dihydrate and 0.5 g of disodium hydrogen citrate sesquihydrate. The mixture was automatically shaken again for 4 min. After centrifugation at 3500 rpm for 5 min, the upper organic phase (5 mL) was separated and transferred to a 15 mL PTFE centrifuge tube which contained 750 mg of anhydrous magnesium sulphate and: (a) 125 mg of PSA; (b) 175 mg of yttria-stabilized zirconium dioxide (the amount of ZrO_2 added was based on previous studies in the literature) or (c) 50 mg of MWCNT. Subsequently, the mixture was shaken for 30 s in a vortex and then centrifuged at 3500 rpm for 5 min. For the comparison with different sorbents the clean-up step was made with a PTFE centrifuge tube which contained 750 mg of anhydrous magnesium sulphate and (a) 175 mg of ZrO_2 nanopowder; (b) 175 mg of Z-sep or (c) 50 mg of GCB. The internal standards used for recovery experiments were carbendazim-d_3 and malathion-d_{10}. As a final step, the upper organic phase was separated, diluted with a mixture of acetonitrile:water and spiked with 10 μL of dimethoate-d_6 at 2.5 μg mL^{-1} (the injection standard) to obtain a concentration of 0.05 mg kg^{-1}; this was subsequently analysed by injecting 5 μL into the UPLC-ESI-QqQ-MS system.

2.4. Analysis by UPLC-ESI-QqQ-MS

For the LC analysis, an Agilent 1290 UPLC system with a binary pump was used. It was equipped with a reversed-phase C8 analytical column of 2.1 mm×100 mm and 1.8 μm particle size (Agilent Zorbax Eclipse plus). Compounds were separated using acetonitrile (mobile phase B) with 0.1 % formic acid and 5 % of water and water with 0.1 % formic acid (mobile phase A). The flow rate used was kept constant at 0.3 mL/min and the gradient programme was set as follows: 20 % B (initial conditions) was kept constant for 2 min followed by a linear gradient up to 100 % B in 4 min, after which the mobile phase composition was maintained at 100 % B for 2 min. The re-equilibration time was 2.5 min. The injection volume was 5 μL. For the mass spectrometric analysis, a 6490 QqQ MS/MS system (Agilent Technologies, Palo Alto, CA, USA) equipped with an electrospray ionization source (ESI), operating in positive ionization mode, was applied using DMRM (dynamic multi-reaction monitoring) software features. The ESI source settings were: gas temperature, 120 °C; gas flow, 13 L/min; nebulizer gas, 45 psi; sheath gas temperature, 375 °C; sheath gas flow, 10 L/min; capillary voltage, 3000 V. Nitrogen was used as the nebulizer and collision gas. Mass Hunter Data Acquisition; Qualitative Analysis and Quantitative Analysis software (Agilent Technologies, Palo Alto, CA, v.B.02) was used for method development and data acquisition. The optimization of the precursor ions, product ions and spectrometric parameters was carried out by the injection of 2 μL of the individual pesticide standard solutions at 0.1 mg L^{-1} in acetonitrile:water (50:50) directly into the mass spectrometer into a constant flow of acetonitrile/water (50/50) with a flow rate of 0.3 ml min^{-1}. These parameters, including the fragment and collision energy (CE), were studied individually. The greatest sensitivity in multiple-reaction monitoring operation mode was achieved through the acquisition of single reaction monitoring (SRM) transitions under DMRM conditions with a 60 s time window. For the identification of analytes, the EU SANCO guidelines for LC–MS/MS analysis were considered [29]. The values for the optimised parameters and the selected SRM transitions in the analytical method are shown in Table 1. For identification purposes, the acquisition of two SRM transitions, the retention time (a tolerance of ±0.2 min) and the SRM ratio compliance (the relationship between the abundance of transitions selected for identification and for quantification, SRM_2/SRM_1, with a tolerance of ±30 %) are needed. A total ion chromatogram with the MRM transitions in the orange and pear extracts at 10 μg kg^{-1} is shown in Fig. 1.

2.5. Analysis by LC-QTOF-MS

To evaluate the amount of matrix compounds in the final extract, a LC-QTOF (6550 Accurate Mass QTOF-MS, Agilent Technologies, Santa Clara, CA) was employed. A QTOF spectrometer, working in full-scan mode, can detect a large number of compounds [30]. Samples were injected into the LC-QTOF and analysed in full-scan mode. The objective of the experiment was to compare the effectiveness of the yttria-stabilized zirconium dioxide as the clean-up sorbent to the typical QuEChERS clean-up sorbent (PSA). In order to obtain the number of compounds present in each of the extracts, data were processed with MassHunter software.

Table 1. Optimized parameter values for the developed method by LC-QqQ-MS/MS.

Compound	t_R(min)	Fragmentor (V)	SRM1	CE1 (V)	SRM2	CE2 (V)
Carbaryl	5.25	380	202.0/127.0	20	202.0/145.0	10
Carbendazim	1.24	380	192.0/160.0	15	192.0/132.0	20
Carbendazim-d_3	1.23	380	195.1/160.0	20	195.1/132.0	20
Chlorpropham	6.08	380	214.0/172.0	5	214.0/154.1	20
Dimethoate-d_6	3.99	380	236.0/205.0	4	236.0/131.0	16
Diphenylamine	6.19	380	170.1/93.1	32	170.1/65.0	36
Ethoxyquin	5.07	380	218.4/173.9	30	218.4/160.2	40
Flutriafol	5.21	380	302.1/95.1	56	302.1/70.1	16
Imazalil	4.67	380	297.0/255.0	15	297.0/159.0	20
Iprodione	6.13	380	330.1/287.9	10	330.1/245.0	15
Malathion-d_{10}	6.15	380	341.1/132.0	12	341.1/100.0	24
Methomyl	2.07	380	163.1/106.0	4	163.1/88.0	0
Flutriafol	5.21	380	302.1/95.1	56	302.1/70.1	16
Imazalil	4.67	380	297.0/255.0	15	297.0/159.0	20
Iprodione	6.13	380	330.1/287.9	10	330.1/245.0	15
Malathion-d_{10}	6.15	380	341.1/132.0	12	341.1/100.0	24
Methomyl	2.07	380	163.1/106.0	4	163.1/88.0	0
Myclobutanil	5.92	380	289.2/125.1	20	289.2/70.2	15
Pirimiphos-methyl	6.53	380	306.2/164.2	20	306.2/108.2	20
Prochloraz	5.59	380	376.0/266.0	15	376.0/70.1	24
Pyrimethanil	4.92	380	200.0/183.0	20	200.0/107.0	20
Thiabendazole	1.31	380	202.0/175.0	30	202.0/131.0	40
Thiophanate-methyl	4.93	380	343.0/151.0	20	343.0/93.0	56
Tolclofos-methyl	6.59	380	300.9/269.0	10	300.9/125.0	15

Table 2. Recoveries % (RSD) at 10 and 100 μg kg^{-1} (n=5) in the two matrices for the method using yttria-stabilized zirconium dioxide as sorbent.

	Orange				Pear			
	10 μg kg^{-1}		100 μg kg^{-1}		10 μg kg^{-1}		100 μg kg^{-1}	
Compound	**Rec. (%)**	**RSD (%)**	**Rec. (%)**	**RSD (%)**	**Rec. (%)**	**RSD (%)**	**Rec. (%)**	**RSD (%)**
Carbaryl	101	2	103	2	106	3	106	2
Carbendazim	84	1	91	5	105	2	109	2
Carbendazim-d_3	83	5	88	1	92	3	99	2
Chlorpropham	–	–	108	7	–	–	105	4
Diphenylamine	104	3	110	2	105	2	105	4
Ethoxyquin	117	3	109	6	–	–	82	1
Flutriafol	98	5	99	5	85	4	84	5
Imazalil	90	5	100	1	89	1	96	0
Iprodione	101	4	109	1	108	5	107	4
Malathion-d_{10}	112	6	112	2	104	6	100	3
Methomyl	89	2	98	3	104	4	105	1
Myclobutanil	99	3	104	4	103	3	108	1
Pirimiphos-methyl	114	1	120	1	104	4	110	0
Prochloraz	100	1	101	1	94	3	99	1
Pyrimethanil	101	2	104	2	102	3	107	1
Thiabendazole	77	0	85	1	96	1	90	1
Thiophanate-methyl	107	1	93	2	43	6	60	2
Tolclofos-methyl	102	3	105	3	93	5	105	6

2.6. Method validation

Mean recovery, linearity, precision (as repeatability and reproducibility, RSD), matrix effects and quantitation limits (LOQ) were established to determine the accuracy and precision of the LC-ESI–MS/MS method following the SANCO guideline on analytical quality control and validation procedures [29]. Linearity was evaluated by

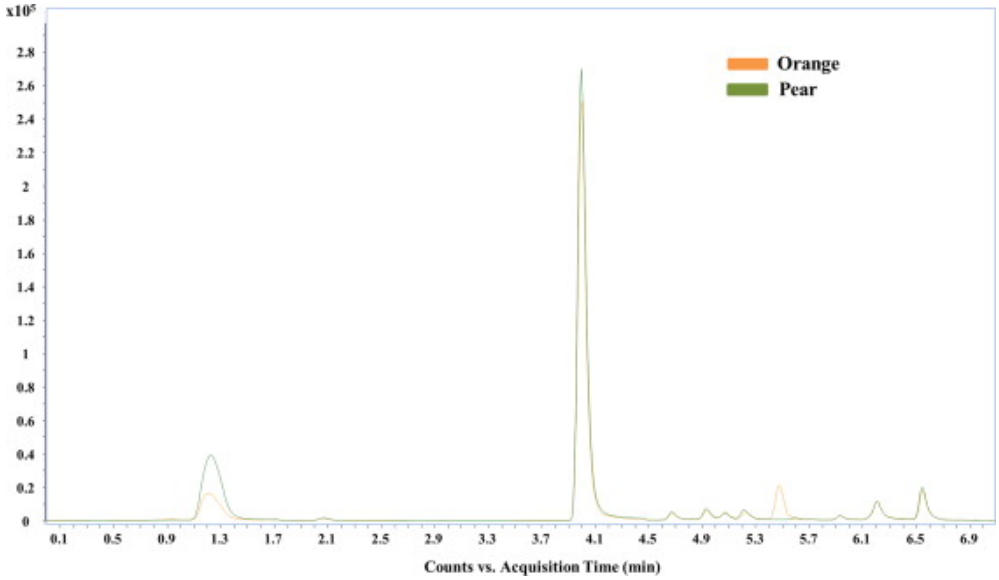

Figure 1. LC-QqQ-MS/MS total ion chromatograms of orange and pear extracts spiked at 10 µg kg^{-1}.

assessing the signal responses of the target analytes from matrix-matched calibration solutions prepared by spiking blank extracts at seven concentration levels, from 0.1 to 50 µg L^{-1}; which, in the sample, corresponded to 1–500 µg kg^{-1} given the 10-times dilution factor. The recoveries and precision of the extraction method were determined as the average of five spiked matrix blanks analysed at concentration levels of 10 and 100 µg kg^{-1}. The precision of the method (represented as the relative standard deviation, RSD %) was obtained from the repeated injection (five times) of a spiked extract at 5 and 50 µg kg^{-1} concentration levels. Precision is expressed as the RSD (%) of the intra-day and inter-day analyses (n=5) over 1 and 5 days, respectively. The method-LOQ should be the lowest validation spiked level meeting this criterion. The LOQ was set as the minimum concentration that can be quantified with acceptable accuracy and precision, as described in the guidance document [29]. For the assessment of matrix effects, standard calibration curves prepared in orange and pear extracts between 1–500 µg kg^{-1} were compared. Furthermore, matrix-matched calibration curves were used for quantitative determinations in order to minimize any ion suppression/enhancement effects: a consequence of the presence of sample matrix components.

2.7. Method application

In order to study the applicability of the method, 20 different samples (10 orange and 10 pear samples) were purchased in various local Spanish shops and then analysed. All samples were stored under the recommended conditions prior to use.

3. Results

3.1. Method validation

The optimization of the method allowed us to meet the recovery residue requirements within the 70–120 % range and with an RSD ≤ 20 % [29] making the use of yttria-stabilized zirconium dioxide a suitable alternative d-SPE material to PSA. Method performance characteristics were evaluated and compared in terms of recovery, quantification limits (LOQs), linearity (r^2), matrix effects and intra-day and inter-day precision. The results obtained in the method performance experiments for determining the 18 pesticides in orange and pear using the proposed QuEChERS method were are summarized in this section.

3.1.1. Recovery study

Recovery studies were carried out at two fortification levels, 10 µg kg^{-1} and 100 µg kg^{-1} in both matrices for the method using yttria-stabilized zirconium dioxide as sorbent. In the orange matrix, at the 100 µg kg^{-1} level, all the pesticides (18 in total) had recoveries within the 70–120 % range (Table 2). The same occurred at the 10 µg kg^{-1} level, except for chlorpropham, which had lower sensitivity at this concentration level-showing a very low detectable S/N ratio. In pear, at the 100 µg kg^{-1} level, 17 pesticides had recoveries within the 70–120 % range; whilst at the 10 µg kg^{-1} level, there were 15. In the case of thiophanate-methyl, low recoveries were obtained at both concentration levels, 60 % and 43 %, respectively, as a result of it degrading into carbendazim. Some other authors have reported low thiophanate-methyl recoveries in various matrices using the QuEChERS extraction method due to degradation [31]. In the case of pear matrix, the pesticide ethoxyquin did not show a recovery value at any concentration level. The problem with ethoxyquin was its low extractability because of the presence of compounds in the pear matrix that interacted with it thus limiting extraction. For example, it has been reported that polyphenol compounds such as phenolic acid or flavonoids, are frequently present in pear and apple [32]. In combination with dilution, the possible solution to degradation problems during sample preparation is maintaining low pH throughout the procedure [33] –

ethoxyquin is only recovered when the sample is acidified in the extraction to pH 3. In both matrices, RSD values (n=5) were below 10 % for all pesticides. The recovery values and RSDs are presented in Table 2.

Comparing this procedure to the QuEChERS method using the typical PSA d-SPE sorbent, at 100 μg kg^{-1} in orange matrix, all of the 18 pesticides had recoveries within the 70–120 % range. However, at the 10 μg kg^{-1} concentration level, ethoxyquin and tolclofos-methyl had lower recovery values (33 % and 55 %, respectively). With respect to the pear matrix, a similar situation occurred when compared with the yttria-stabilized zirconium dioxide sorbent method. At the 100 μg kg^{-1}, 16 pesticides had recoveries within the 70–120 % range. The two pesticides with the lowest recoveries were ethoxyquin and thiophanate methyl. At 10 μg kg^{-1}, there were 15 pesticides with recoveries within the 70–120 % range. Recovery value results are not shown in Table 2.

Results obtained when using MWCNT as the d-SPE sorbent showed that at 10 μg kg^{-1} in orange matrix only 13 pesticides had recoveries within the 70–120 % range and at 50 μg kg^{-1}, there were 15 pesticides. In pear matrix, recoveries were even worse, at 10 μg kg^{-1}, only 10 pesticides had recoveries within the 70–120 % range and at 50 μg kg^{-1} there were 11 pesticides. The pesticides with the lowest recoveries were carbendazim, prochloraz, pyrimethanil, thiabendazole and thiophanate-methyl. It has been reported previously that graphitized carbon black (GCB) and other carbon-based sorbents retain pesticides with planar structures such as carbendazim and thiabendazole resulting in poor recovery and precision [34] and [35]. As a result, the QuEChERS method using MWCNT as the d-SPE sorbent is not suitable for the recovery of these pesticides and, therefore, it was not selected for further validation studies.

Considering the results obtained comparing the standard QuEChERS procedure with the various clean-up methods, it was demonstrated that yttria-stabilized zirconium dioxide can be used as an effective d-SPE material with the QuEChERS method as a suitable alternative material to PSA for the extract clean-up in orange and pear matrices. For this reason, it was selected for further validation studies.

3.1.2. Limits of quantification

Quantification limits obtained from the validation of the two modified QuEChERS extraction methods developed in this study were 10 μg kg^{-1} for almost all pesticides in the orange matrix, except from chlorpropam, which was 100 μg kg^{-1}. In the case of pear matrix, LOQs were 10 μg kg^{-1} for almost all pesticides, except from chlorpropam and ethoxyquin, which were 100 μg kg^{-1}; and in the case of thiophanate-methyl, the limit was 100 μg kg^{-1} but with recoveries below 70 %. The LOQs are shown in Table 3 for all the pesticides selected in the study.

3.1.3. Linearity

Linearity was studied in the 1–500 μg kg^{-1} range, corresponding to 0.1–50 μg L^{-1} (given the 10-times dilution factor) for all the pesticides at seven calibration levels (1, 2, 5, 10, 50, 100 and 500 μg kg^{-1}) by matrix-matched standard calibration in blank orange and pear extracts. Linearity values calculated as correlation coefficients for each pesticide from the matrix-matched calibration (yttria-stabilized zirconium dioxide clean-up) plots are shown in Table 3. The quantitative results of a detection method greatly depend on its calibration method. Good linearity was found for most of the pesticides with r^2 values higher than 0.99, and both the pure solvent-based as well as the matrix-matched gave r^2 values better than 0.99, ranging from 0.9974–1.0000 in orange and 0.9969–1.0000 in pear, respectively. This is remarkable considering the complexity of matrices such as orange. Concerning the linear range for the two matrices, in most cases, this started at a concentration of 1 or 2 ppb and the detector response was linear up to 500 μg kg^{-1}. This range is also presented in Table 3.

3.1.4. Inter- and intra-day precision

Precision values for the chromatographic method, presented as intra- and inter-day variability, were calculated as % RSD; for all the analytes, these ranged from 1–12 % for intra-day (n=5) and from 0-16 % for inter-day (over 5 days). The RSD ≤20 % criterion, recommended by DG-Sanco guidelines [29] was met for all compounds in both matrices.

3.1.5. Matrix effects

Matrix effects were evaluated by comparing the response of pesticide standards, prepared in orange and pear blank extracts, with standards in solvent. The response in orange extracts was substantially lower because of ionization suppression from coextracted matrix components. A dilution of the sample extracts with a mixture of acetonitrile/water was assayed in order to reduce these interferences. The results obtained for five-fold diluted extracts were better; and for ten-fold diluted extracts were even better still; however, the average signal was still approximately half that of the solvent standards for some pesticides. In order to compensate for the matrix effect, matrix-matched calibration was required using blank extracts diluted ten-fold with a mixture of acetonitrile/water. The matrix effect values are presented in Table 3. For orange, 8 pesticides exhibited soft matrix effects (suppression or enhancement of 0–20 %), 4 medium (suppression or enhancement of 20–50 %) and 6 showed strong matrix effects (suppression or enhancement >50 %). In the pear matrix, all 18 pesticides exhibited soft matrix effects.

The effectiveness of yttria-stabilized zirconium dioxide as the d-SPE sorbent can be assessed by evaluating the

amounts of matrix compounds in the final extract using a LC-QTOF-MS/MS working in full-scan mode. Two orange extracts were evaporated, reconstituted in acetonitrile and water (20 % acetonitrile) and diluted five times. In the first case, acetonitrile extract was cleaned with $MgSO_4$ and PSA whilst in the second, $MgSO_4$ and yttria-stabilised zirconium dioxide were used. To obtain the number of compounds present in each of the extracts, the data were processed with MassHunter software. As can be seen in Fig. 3, in orange, 8647 compounds were present in the extract prepared with the typical QuEChERS clean-up sorbent (PSA) whereas in the extract treated with yttria-stabilized zirconium dioxide, there were 7938. We can also evaluate the results by comparing the full-scan chromatograms with the two different clean-up methods (Fig. 2). The orange extract chromatograms are

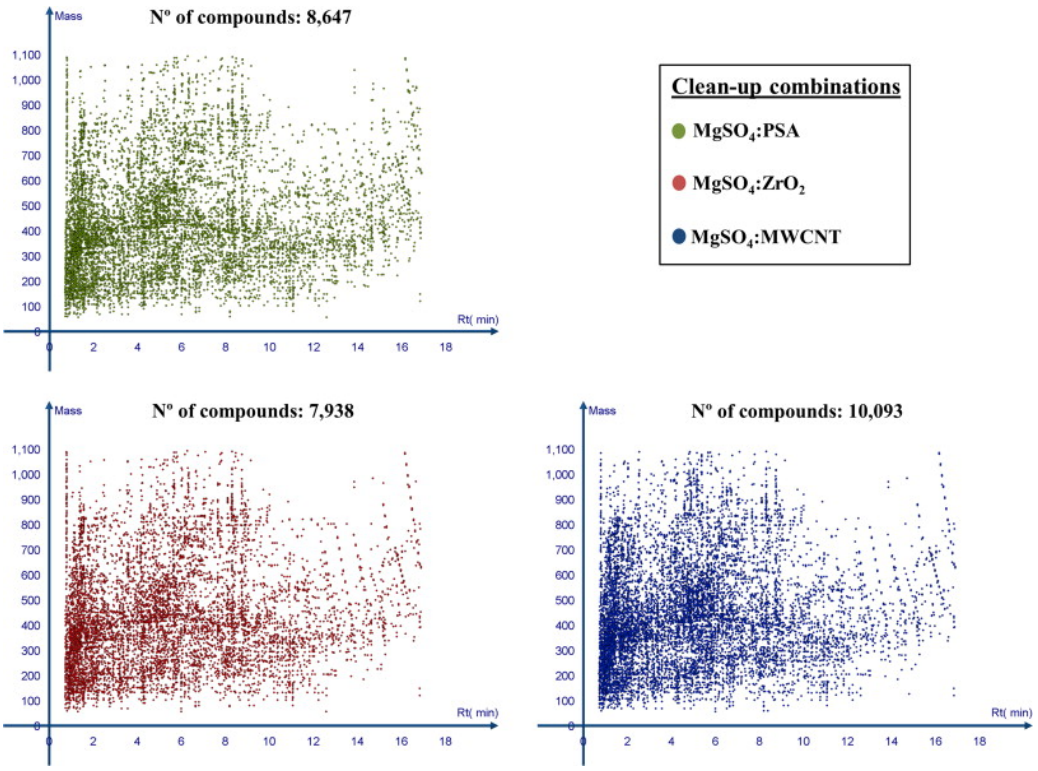

Figure 2. LC-QToF-MS full-scan chromatograms of blank orange and pear extracts diluted 5-times obtained by using QuEChERS methodology and two different clean-up procedures.

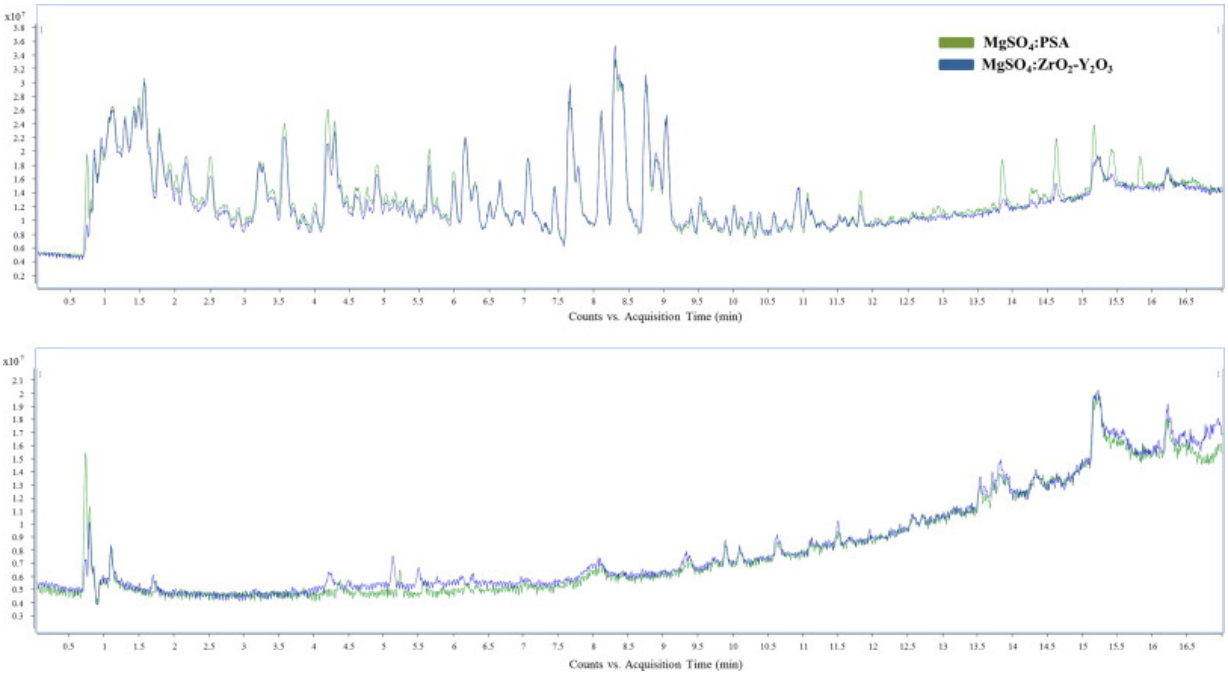

Figure 3. Co-eluting matrix compounds of orange extracts analysed using LC-QTOF-MS with the different clean-up procedures (absolute height 10,000 counts). The *x*-axis represents the retention time and the *y*-axis the *m*/*z*.

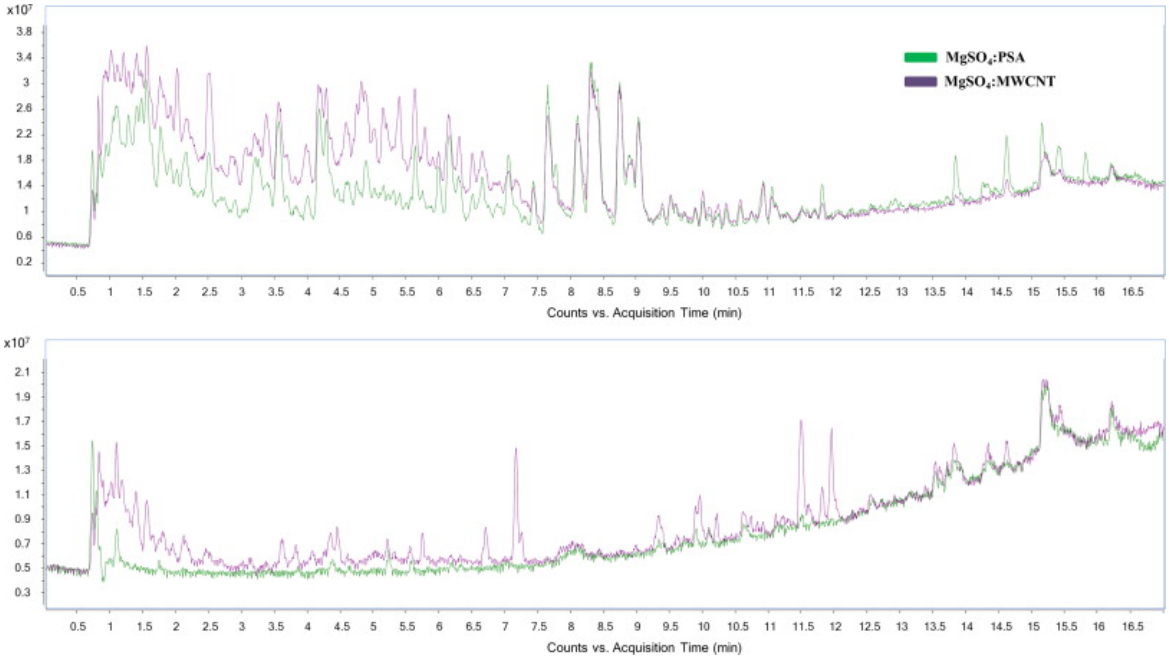

Figure 4. LC-QToF-MS full-scan chromatograms of blank orange and pear extracts diluted 5-times obtained by using QuEChERS methodology and two different clean-up procedures.

Table 3. Limits of quantification, concentration range and matrix effects for the selected matrices studied.

Compound	**LOQ (μg kg^{-1})**		***r*^{2}**		**Instrumental concentration range (μg kg^{-1})**		***ME (%)***	
	Orange	**Pear**	**Orange**	**Pear**	**Orange**	**Pear**	**Orange**	**Pear**
Carbaryl	10	10	0.9992	0.9997	2–500	1–500	−56	−7
Carbendazim	10	10	0.9994	0.9999	1–500	1–500	−55	−10
Carbendazim-d_3	10	10	0.9991	0.9999	1–500	1–500	−53	−9
Chlorpropham	100	100	0.9996	1.0000	50–500	50–500	−5	−3
Diphenylamine	10	10	0.9989	0.9998	1–500	1–500	−4	−4
Ethoxyquin	10	100	0.9982	0.9969	10–500	100–500	−10	−1
Flutriafol	10	10	1.0000	0.9997	1–500	1–500	−50	−12
Imazalil	10	10	0.9995	0.9999	1–500	1–500	−19	−6
Iprodione	10	10	0.9992	0.9992	10–500	10–500	2	14
Malathion-d_{10}	10	10	0.9998	0.9989	1–500	1–500	23	4
Methomyl	10	10	0.9998	1.0000	1–500	1–500	−12	−7
Myclobutanil	10	10	0.9974	0.9993	1–500	1–500	−1	−4
Pirimiphos-methyl	10	10	0.9996	0.9997	1–500	1–500	−14	−6
Prochloraz	10	10	0.9987	0.9996	1–500	1–500	−51	−4
Pyrimethanil	10	10	0.9997	1.0000	1–500	1–500	−29	−4
Thiabendazole	10	10	0.9989	0.9996	1–500	1–500	−55	−7
Thiophanate-methyl	10	100[a]	0.9975	0.9969	1–500	1–500	46	1
Tolclofos-methyl	10	10	0.9999	1.0000	5–500	5–500	−27	−10

[a] In this case the recovery was 60%.

practically identical, apart from differences in the first part of the chromatogram for the method based on yttria-stabilised zirconium dioxide where the signal for some pesticides is lower than in the other method. The same evaluation using LC-QTOF-MS/MS was carried out for the MWCNTs to evaluate its effectiveness. Although orange and pear extracts obtained after clean-up with MWCNT sorbent seems to be cleaner that with classical PSA sorbent, it has been demonstrated that 8647 compounds were present in the extract prepared with the typical QuEChERS clean-up sorbent (PSA) whereas in the extract treated with MWCNT, there were 10,093. Fig. 4 shows the results comparing the full-scan chromatograms for orange and pear extracts obtained with the two different clean-up methods.

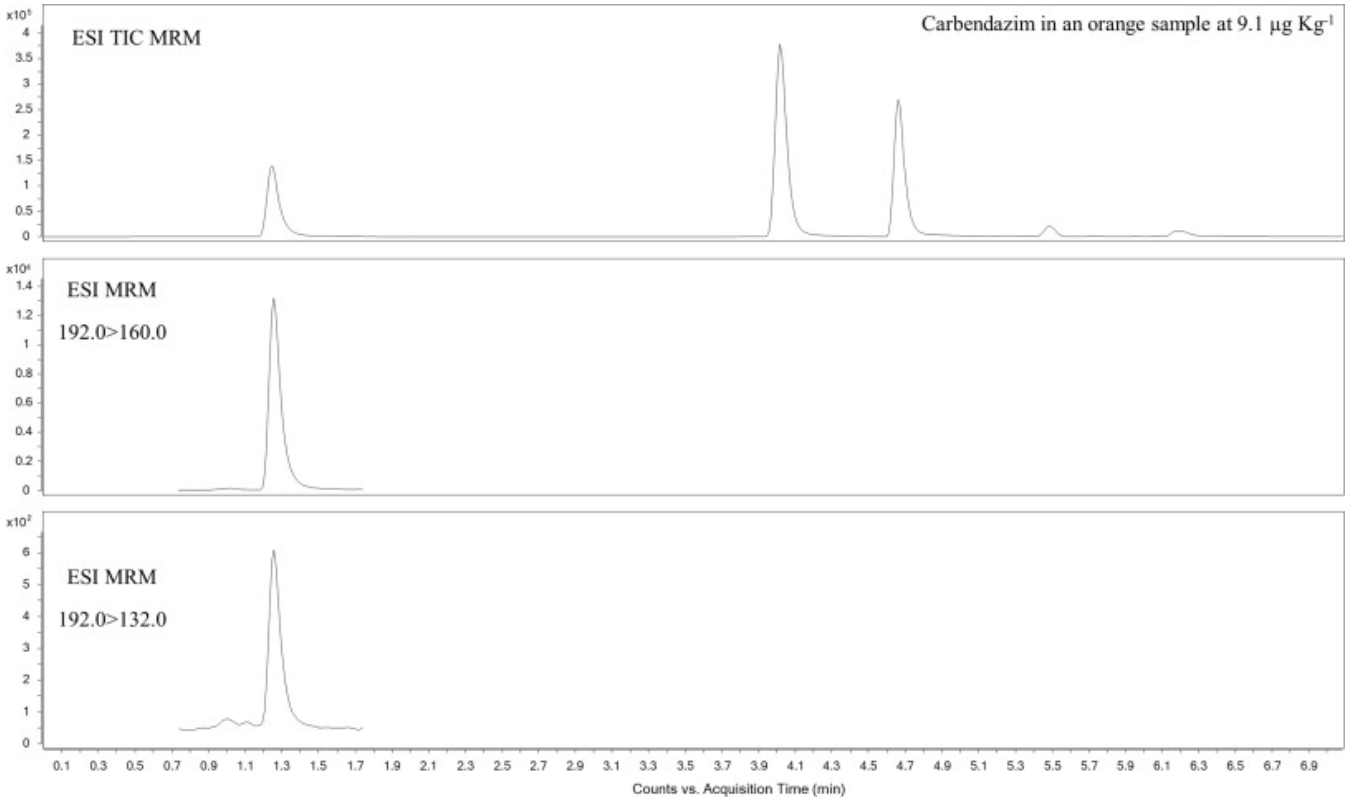

Figure 5. Total ion chromatogram and extracted ion chromatograms of the two transitions of carbendazim in an orange sample at 9.1 µg kg^{-1}.

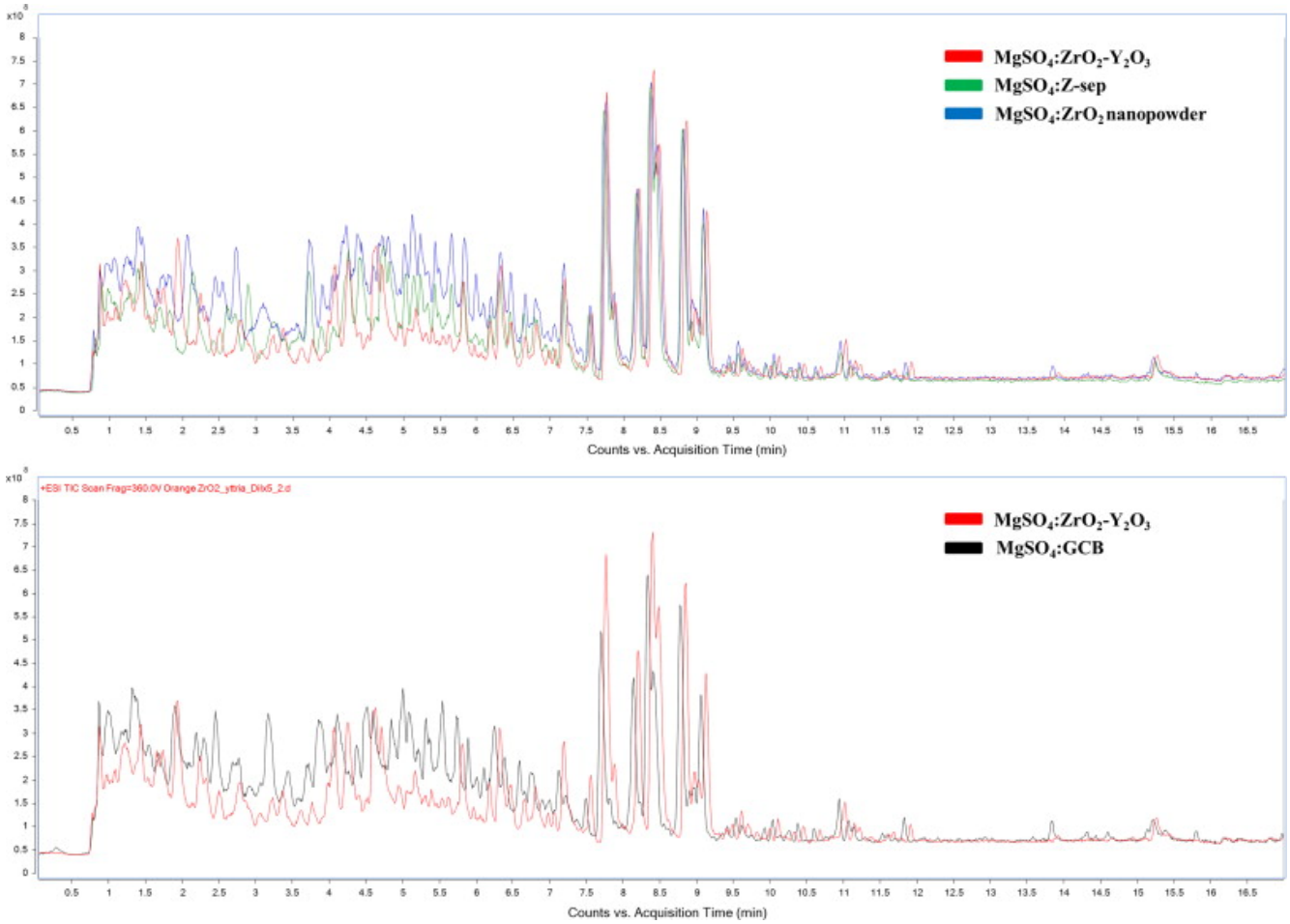

Figure 6. LC-QToF-MS full-scan chromatograms of blank orange extracts diluted 5-times obtained by using QuEChERS methodology with yttria-stabilized zirconium dioxide, zirconium dioxide nanopowder, Z-sep and GCB clean-up sorbents.

3.1.6. Comparison with other ZrO_2 based sorbents and with GCB

In order to study the effectiveness of yttria-stabilised zirconium dioxide as d-SPE sorbent, others as ZrO_2 nanopowder of less surface area, Z-sep and graphitized carbon black have been used and compared in terms of recoveries and number of matrix compounds. Recovery studies were carried out at 10 μg kg^{-1} fortification level in orange matrix. As has been previously discussed, for the method using yttria-stabilized zirconium dioxide as sorbent, 17 pesticides had recoveries within the 70–120 % range (chlorpropham had lower sensitivity at this concentration level). Otherwise, for the method using ZrO_2 nanopowder of less surface area, 16 pesticides had recoveries within the 70–120 % range. The pesticide with the lowest recovery was pirimiphos-methyl (60 %). Alternatively, for the method using Z-sep, 14 pesticides had recoveries within the 70–120 % range. The three pesticides with the lowest recoveries were pirimiphos-methyl (61 %), prochloraz (63 %) and thiabendazole (63 %). Finally, for the method using GCB, 12 pesticides had recoveries within the 70–120 % range. The five pesticides with the lowest recoveries were carbendazim (and, therefore, its deuterated analog) (53 %), pirimiphos-methyl (61 %), pyrimethanil (65 %) and thiabendazole (22 %). It is well known that a significant loss of pesticides with planar ring structures as thiabendazole, is caused by its strong retention in graphitized carbon black (GCB) in dispersive SPE [36] and [37]. An evaluation of the number of coextractives obtained with the different sorbents using LC-QTOF-MS/MS was made and data were processed with MassHunter software. Orange extracts were evaporated, reconstituted in acetonitrile and water (20 % acetonitrile) and diluted five times. In orange, 7757 compounds were present in the extract prepared with yttria-stabilized zirconium dioxide clean-up sorbent whereas in the extract treated with ZrO_2 nanopowder, Z-sep and GCB, there were 8949, 7025 and 8698, respectively. We can also evaluate the results by comparing the full-scan chromatograms with the three different clean-up methods using ZrO_2 based sorbents and with GCB method (Fig. 6). The orange extract chromatograms are practically identical for the yttria-stabilized zirconium dioxide sorbent and for Z-sep, apart from differences in the first part of the chromatogram for the method based on ZrO_2 nanopowder where the signal for some pesticides is higher than in the other methods. With respect to the comparison with GCB, there are more differences in the chromatograms; the signal for some pesticides is much higher with the GCB than for yttria-stabilized zirconium dioxide sorbent.

According to the data obtained, it has been proved that yttria-stabilized zirconium dioxide can be used as an effective d-SPE material with the QuEChERS method acting as a suitable alternative material to such different sorbents as PSA, other ZrO_2 based sorbents or even GCB in the extract clean-up of difficult matrices such as oranges.

3.2. Method application

The developed QuEChERS method with the ZrO_2-Yttria clean-up step was applied to real samples. Twenty fruit samples (including oranges and pears) from local Spanish supermarkets were treated with the sample preparation method described in the experimental section and analysed by LC–MS/MS. Four different pesticide residues were detected in some of these commodities (Table 4). Twenty percent (4 out of 20) of the samples contained pesticide residues at a quantifiable level (equal to or above the LOQs) for at least one pesticide residue. The typical post-harvest fungicides used in citrus crops were detected in the samples-specifically: imazalil was found in orange 6 (1007 μg kg^{-1}), imazalil and pyrimethanil were found in pear 2 (755 μg kg^{-1} and 893 μg kg^{-1}), carbendazim and imazalil were found in pear 3 (64 μg kg^{-1} and 1175 μg kg^{-1}) and imazalil was found in pear 4 (15 μg kg^{-1}). The most frequently-detected pesticide residues, independent of the commodity, were: carbendazim (50 %), thiabendazole (50 %) and imazalil (35 %). These two post-harvest pesticides (carbendazim and thiabendazole) were detected in many of the samples analyzed but in almost all cases below 10 μg kg^{-1}. The highest pesticide residue concentrations were for imazalil-found in orange at 1007 μg kg^{-1} and in pear at 1175 μg kg^{-1}. These results are in agreement with others reported in the literature, where the majority of the orange samples analysed contained imazalil and thiabendazole residues [38], [39], [40] and [41]. It is important to note that all detected pesticides were below the MRL established by European MRL regulations [6]. All the detections were confirmed by the second transition selected, obtaining an ion ratio within the accepted tolerance (±30 %) in all cases [29]. An example of a positive sample, carbendazim in orange number 6 at 9.1 μg kg^{-1} is shown in Fig. 5.

Table 4. Pesticide residues detected in fruit samples.

	Carbendazim	Imazalil	Pyrimethanil	Thiabendazole
Orange				
1	<LOQ	nd	nd	<LOQ
2	<LOQ	nd	nd	<LOQ
3	<LOQ	<LOQ	<LOQ	<LOQ
4	<LOQ	<LOQ	<LOQ	<LOQ
5	<LOQ	nd	<LOQ	<LOQ
6	<LOQ	1006.9	<LOQ	<LOQ
Pear				
1	<LOQ	<LOQ	<LOQ	<LOQ
2	<LOQ	755.2	892.6	<LOQ
3	63.9	1175.4	nd	<LOQ
4	<LOQ	14.5	nd	<LOQ

Concentration: μg kg^{-1}; nd: not detected. <LOQ: detected but at lower concentration. (<10 μg kg^{-1}).

4. Conclusions

In this work, a very quick, easy, effective, reliable and accurate multi-residue method based on a modified

QuEChERS extraction method was developed for the determination of pesticides in fruits by LC–QqQ-MS/MS. We demonstrated that yttria-stabilized zirconium dioxide can be used as an effective d-SPE material with the QuEChERS method acting as a suitable alternative material to PSA in the extract clean-up of various matrices; however, MWCNTs did not prove useful as clean-up sorbents for these matrices. The method's validation parameters in terms of recovery, quantification limits (LOQs), linearity (r^2), matrix effects and intra-day and inter-day precision showed that the proposed method meets the pesticide analysis requirements (average recovery values were in the 77–120 % range for almost all selected pesticides with RSD values lower than 10 %). Yttria-stabilized zirconium dioxide has proven to be a new type of d-SPE sorbent material and is expected to be widely applied for pesticide analysis at trace levels in future sample clean-up.

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