Automatisation of the clean-up step of multiresidue methods in GC-MS

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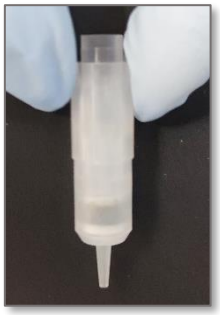
1. Aim and scope

This document reports the implementation of an automated µSPE clean-up method and its comparison with manual dispersive clean-up strategies for the analysis of pesticide residues in different matrices by gas chromatography, as a way to increase the sample throughput while minimizing the experimental work.

2. Short description

The clean-up step is essential during the extraction process to remove undesired matrix components that may cause analytical interferences, especially in gas chromatography. However, this additional procedure entails time-consuming work and, moreover, it usually needs to be adapted to different matrices (for instance, matrices with a high oil content might need a freezing-out step to remove the lipid content, while others only need a solid sorbent). Therefore, the development of an automated and unified clean-up procedure would result in a significant reduction of the time and laboratory work needed for pesticide residue analysis.

In the present work, five different matrices including high water content, high acid, high fat or dry material have been extracted following either manual dispersive clean-up (different procedures according to the matrix group) or an automated µSPE clean-up workflow using a PAL RTC instrument. The latter technique employs clean-up cartridges containing a mixture of sorbent materials suitable for multiple matrices followed by direct injection in the GC instrument (*see picture*). All samples were analyzed by gas chromatography and the results of both techniques have been compared in terms of cleanliness of the extracts, recoveries, signal intensity and interferences.



3. Experimental

3.1. Sample treatment

Tomato, orange, tea, rice and olive samples were extracted using the QuEChERS method (one extraction of blank matrix and one extraction of a sample spiked with a mixture of 106 GC-amenable compounds at a concentration of 10 µg/kg). The general experimental procedure was as follows:

1. Weigh 10 g (tomato, orange, olive), 5 grams (rice) or 2 grams (tea) of sample in a 50-mL PTFE centrifuge tube.
2. Add 10 mL H_2O and shake manually for 3 sec (tea and rice).
3. Add 10 mL acetonitrile.
4. Shake the sample in an axial agitator (Agitax) for 6 minutes.
5. Add 4 g anhydrous magnesium sulphate, 1 g sodium chloride, 1 g trisodium citrate dihydrate and 0.5 g disodium hydrogencitrate sesquihydrate and shake manually (3 sec).
6. Shake the sample in an axial agitator (Agitax) for 6 minutes.
7. Centrifuge the tubes at 4000 rpm for 5 min.
8. Take an aliquot of the supernatant for the µSPE experiments.

The clean-up procedure was adapted to each matrix, as described below.

Tomato and orange:

1. Transfer 5 mL of the supernatant to a 15-mL PFTE centrifuge tube containing 750 mg anhydrous magnesium sulphate and 125 mg PSA (primary secondary amine) and vortex for 30 sec.
2. Centrifuge the tubes at 4000 rpm for 5 min.
3. Transfer the supernatant to a 4-mL vial and add 10 µL of a formic acid solution in acetonitrile (5 % volume) per mL of extract.

Rice:

1. Transfer 8 mL of the supernatant to a 15-mL PFTE centrifuge tube and place it in a box containing dry ice for 20 min.
2. Centrifuge the tubes at 4000 rpm for 1 min.
3. Transfer 5 mL of the supernatant to a 15-mL PFTE centrifuge tube containing 750 mg anhydrous magnesium sulphate and 125 mg PSA (primary secondary amine) and vortex for 30 sec.
4. Centrifuge the tubes at 4000 rpm for 5 min.
5. Transfer the supernatant to a 4-mL vial and add 10 µL of a formic acid solution in acetonitrile (5 % volume) per mL of extract.

Olive:

1. Pre-condition EMR-Lipid tubes with 5 mL H_2O (MilliQ) and vortex for 30 seconds.
2. Add 5 mL of QuEChERS extract (supernatant) and vortex for 30 sec.
3. Centrifuge the tubes at 4000 rpm for 5 min.
4. Add 5 mL of supernatant to EMR-Polish tubes and vortex for 30 sec.
5. Centrifuge the tubes at 4000 rpm for 5 min.
6. Transfer the supernatant to a 4-mL vial.

Tea:

1. Transfer 5 mL of the supernatant to a 15-mL PFTE centrifuge tube containing 250 mg calcium chloride and 125 mg PSA and vortex for 30 sec.
2. Centrifuge the tubes at 4000 rpm for 5 min.
3. Transfer the supernatant to a 4-mL vial and add 10 µL of a formic acid solution in acetonitrile (5 % volume) per mL of extract.

For the injection vial preparation, samples with clean-up (**QC extracts**) were evaporated under a gentle N_2 current and reconstituted with ethyl acetate prior to injection; a 10-ppb standard was prepared using the blank extract (reconstitution with the pesticide mix in ethyl acetate). Sample aliquots without clean-up (for **µSPE**) were diluted 1:1 with acetonitrile; a 10-ppb standard was prepared by adding the pesticide mix to the blank extract (1:1).

3.2. Automated µSPE

µSPE cartridges (p/n µSPE-GCQuE1-45 provided by CTC Analytics) were employed to perform an automated clean-up procedure which was then compared to the manual dispersive clean-up methods. The cartridge composition was 45 mg of sorbents with the following composition (weight percentage): 20/12/12/1 anhydrous MgSO4/PSA/C18/CarbonX. Only one type of cartridge was applied to all matrices, conversely to the manual dispersive clean-up methods (**Table 1**). The following simple workflow was designed based on the work by Lehotay *et al.* [1]: first, the cartridges were pre-conditioned with 100 µL acetonitrile prior to sample loading. Then, 200 µL of each sample raw extractswere loaded into the cartridge at 10 µL/sec and the clean extract was collected into a 2-mL vial with a pre-cut septum cap. The samples were afterwards automatically injected in the GC chromatograph and analyzed for pesticide residues.

The whole automated sample treatment and vial injection was performed with the use of a PAL RTC (Robotic Tool Change) coupled to the GC-MS/MS instrument. This type of autosampler is, also available by other manufacturers. The µSPE cartridges have been developed for their use with acetonitrile solvent, so the QuEChERS (without clean-up) extracts are directly used after the 2-fold dilution with acetonitrile (no solvent change) to ensure comparability between samples and calibration standards.

Table 1. Clean-up salts and general procedures for the different matrices in the QC and µSPE method

	QC	µSPE
Olives	EMR-Lipid tubes + EMR-Polish tubes	Mini cartridges containing 45 mg of MgSO4 + PSA + C18 + CarbonX (20:12:12:1)
Orange	$MgSO_4$ + PSA (6:1)	
Tomato		
Tea	$CaCl_2$ + PSA (2:1)	
Rice	Freezing-out (20 min) $MgSO_4$ + PSA (6:1)	

3.3. Analysis by GC-QqQ-MS/MS

All samples were analyzed by an Intuvo 9000 GC Instrument coupled to an 7010B GC/MS Triple Quad (Agilent Technologies). The analytical parameters are detailed below.

- Column: 2 Planar columns HP-5MS UI (15 m long × 0.25 mm i.d. × 0.25 µm film thickness)
- Injection mode: splitless, 1 µL,
- Ultra-inert inlet liner with glass wool frit from Agilent
- Injector temperature: 70 °C (0.1 min), then up to 325 °C at 800 °C/min (hold for 5 min).
- Carrier gas: Helium at constant flow = 1.28 mL/min column 1, 1.48 mL/min column 2.
- Oven temperature: 60 °C for 0.5 min, up to 170 °C (80 °C/min) and up to 310 °C (20 °C/min).
- Ionization mode: electron impact ionization.
- Temperature of the transfer line: 280 °C.
- Temperature of ion source: 280 °C.
- Collision gas: nitrogen.
- Solvent delay: 2.6 minutes.

3.4. Analysis by GC-TOF-MS

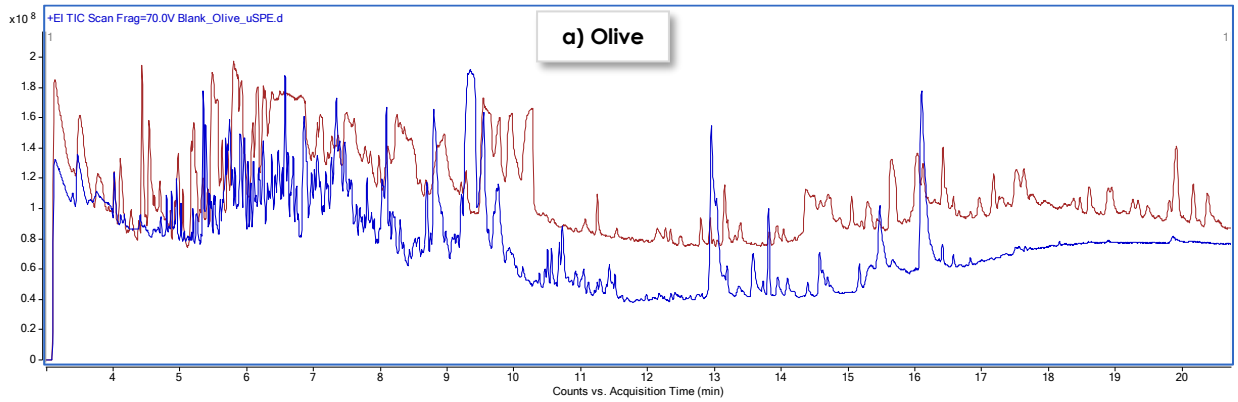
The Total Ion Chromatograms (TICs) were obtained using an Agilent 7890A gas chromatograph. The samples were injected using a multimode injector equipped with an ultra-inert inlet liner, with a glass wool obtained from Agilent. The multimode injector operated in solvent vent mode with a temperature program of 70 °C (0. 1 min), 800 °C/min to 325 °C. The injection volume was 5 µL and helium (99.999 %purity) was used as carrier gas. The instruments used two fused silica HP-5MS UI capillary column of 15 m × 0.250 µm inner diameter and a film thickness of 0.25 µm (Agilent) connected by a capillary flow technology (CFT) union. The oven temperature was programmed as follows: 60 °C for 1 min, 40 °C min^{-1} to 170 °C and finally up to 310 °C at 10 °C min^{-1}. The total run time was 20.75 min with three additional minutes for backflushing at 310 °C. The gas chromatography system was connected to a quadrupole time-of-flight (QTOF) mass spectrometer Agilent 7250 operating in electron impact ionization (EI) mode (70 eV). The ion source and transfer line were set at 280 °C and quadrupole temperature was set at 150 °C. A solvent delay of 3 min was selected in order to prevent damage in the ion source filament. TOF-MS was operated at full scan mode from m/z 60 to 500 and an acquisition rate of 3 spectra/s.

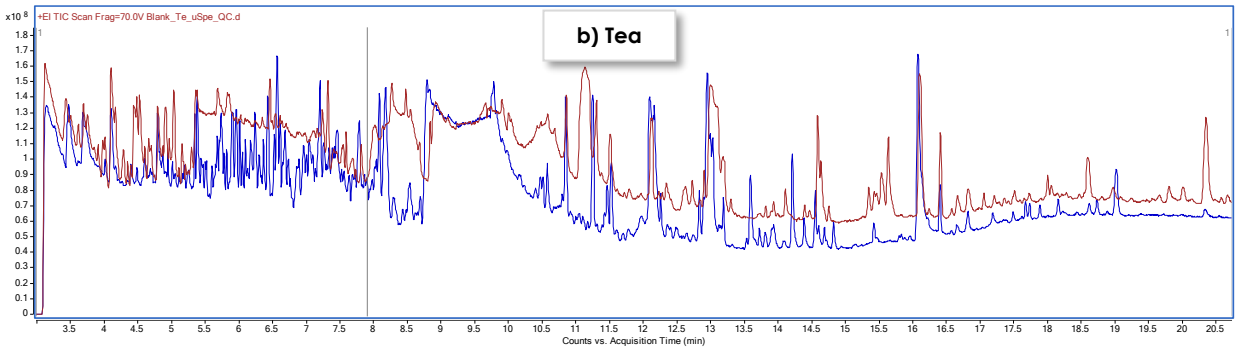
4. Results and discussion

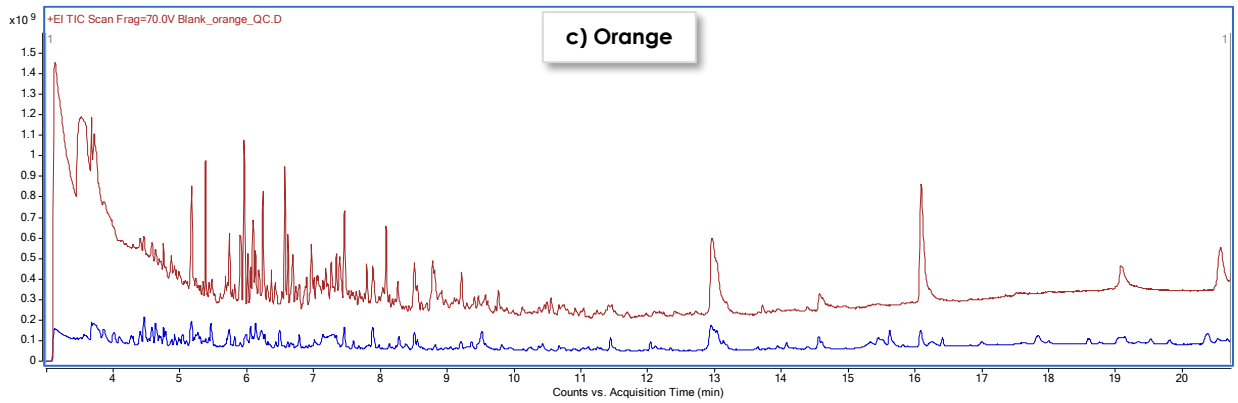
4.1 TICs and extract appearance

As depicted in the TIC chromatograms (**Figure 1**), the baseline obtained with the µSPE method is lower than the QC manual method (original or modified QuEChERS citrate method according to the matrix) for olive, orange and tea, whereas the opposite effect can be seen in tomato and rice matrices. In the latter cases, the samples have a reduced number of matrix components and the use of a µSPE cartridge may result in interferences that do not arise from the matrix itself, but the cartridge components.

The cartridge-derived components can be seen in the last TIC chromatogram, which depicts the solvent (ethyl acetate) before and after passing through the µSPE cartridge. A higher baseline is obtained with the cartridge, plus a number of interferences derived from the cartridge polymers that appear in the first third of the chromatogram. However, most of the pesticide residues are not affected by these interferences, as the majority of the analytes elute later from the chromatographic column.




+EI TIC Scan Frag=70.0V Blank_Te_uSpe_QC.d
b) Tea
Counts vs. Acquisition Time (min)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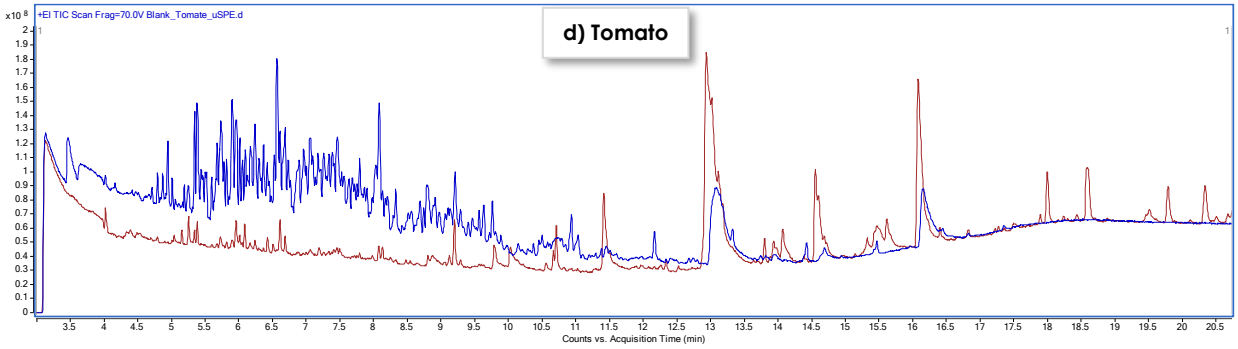
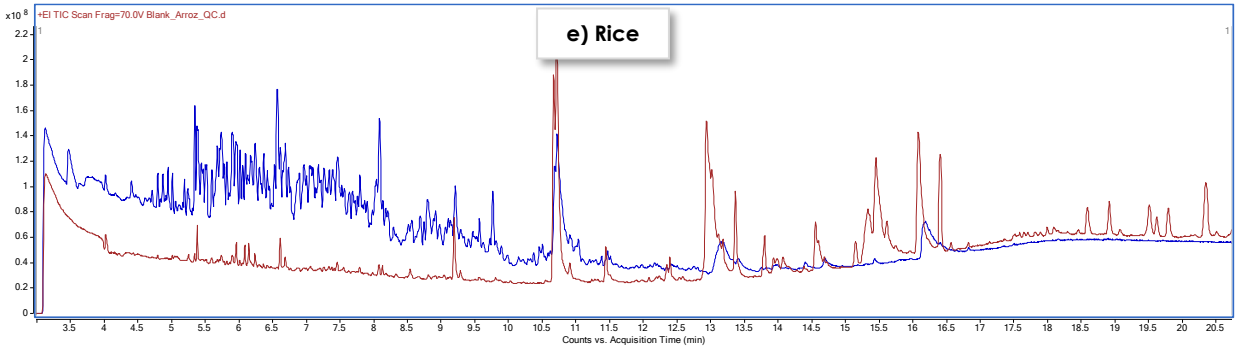
c) Orange
Counts vs. Acquisition Time (min)


+EI TIC Scan Frag=70.0V Blank_Tomate_uSPE.d
d) Tomato
Counts vs. Acquisition Time (min)


+EI TIC Scan Frag=70.0V Blank_Arroz_QC.d
e) Rice
Counts vs. Acquisition Time (min)

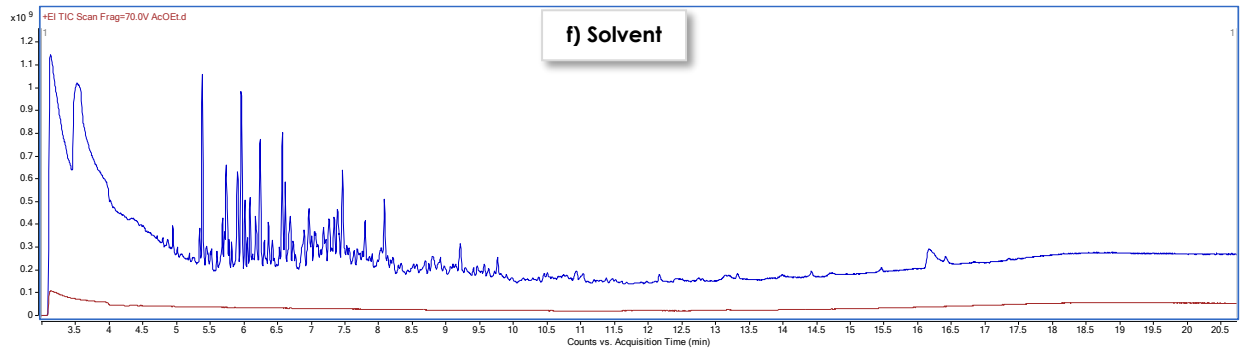


Figure 1. TIC chromatograms obtained with a GC-Q-TOF instrument for the blank extracts from the QC method (dark red color) and µSPE method (blue color)

Regardless of their TICs, a dramatic difference could be seen in all µSPE extracts after the cartridge had been employed. **Figure 2** shows three vials of both tomato and tea samples: QuEChERS extract without clean-up, final extract after manual dispersive clean-up (with $MgSO_4$ or $CaCl_2$ and PSA) and final extract after µSPE clean-up. Only minor differences can be seen between the raw extract and the QC extract (with dispersive clean-up). However, the µSPE extracts were in all cases clear and colorless.

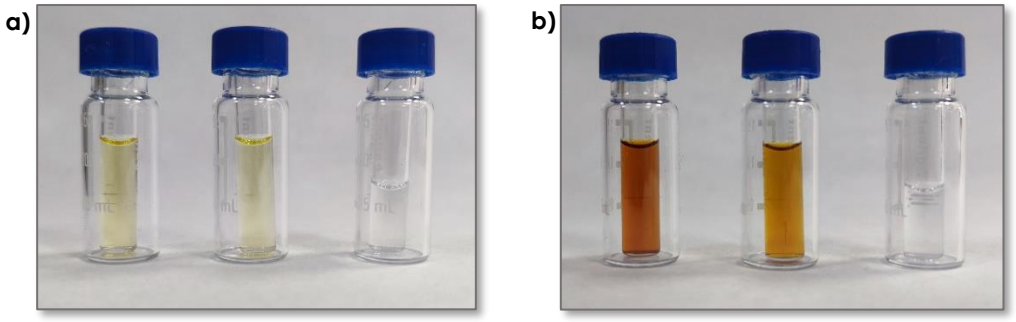


Figure 2. Extracts of (a) tomato and (b) tea samples. From left to right: raw QuEChERS extracts without clean-up, QC extract (dispersive clean-up) and µSPE extract (cartridges)

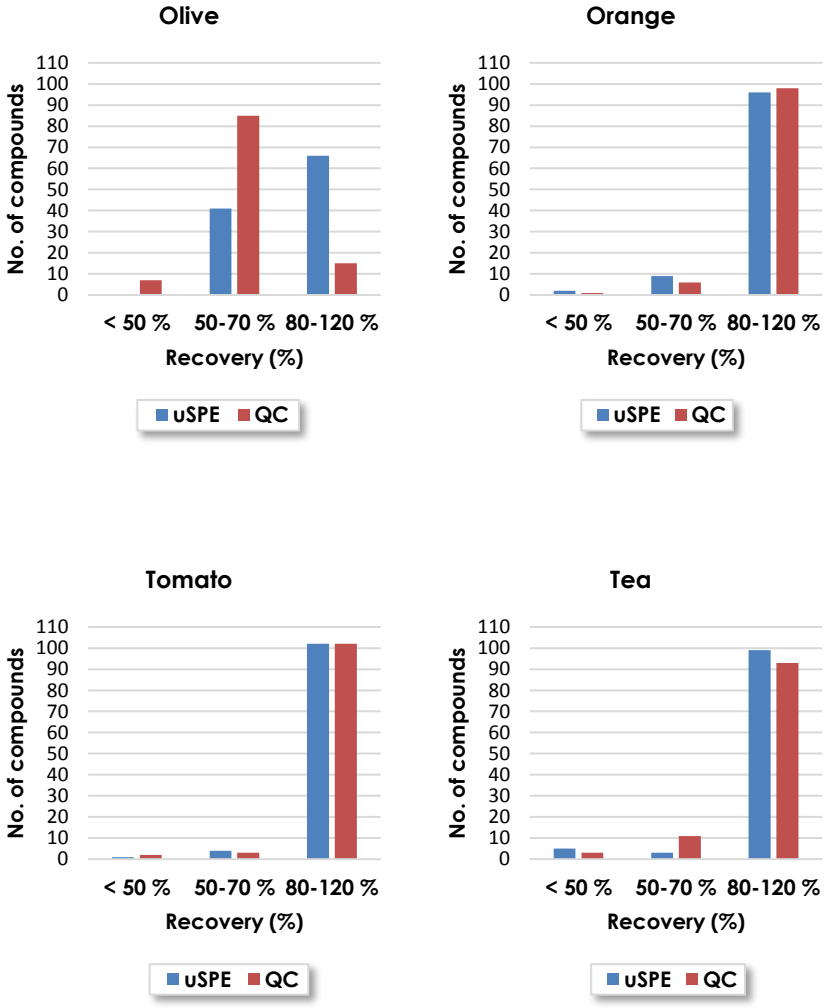
4.2 Recoveries

The average recoveries of each one the matrices included in the present study are depicted in **Table 2**. In general, very similar results were obtained with both techniques, being slightly higher in some cases with µSPE. This approach does not include an evaporation step to change the solvent prior to the injection, and therefore the volatile compounds (which are an important number of the GC-amenable analytes) can reach recoveries close to 100 %. This was the case, for instance, of dichlorvos, which in QC showed recoveries a recovery of 66 % and 69 % in tomato and rice matrices, respectively, while rising up to 98 % in both matrices with the µSPE method.

Figure 3 shows the number of compounds that were in different recovery ranges for the matrices tested in the present study: except for the case of olive, very similar results were obtained for the vast majority of compounds, despite using only one unified type of cartridge for the automated clean-up instead of specific salts for each matrix group.

Table 2. Average recovery (%) of 106 compounds in different matrices with µSPE and QC method

	µSPE	QC
Olives	82	70
Orange	91	97
Tomato	99	98
Tea	99	98
Rice	103	91



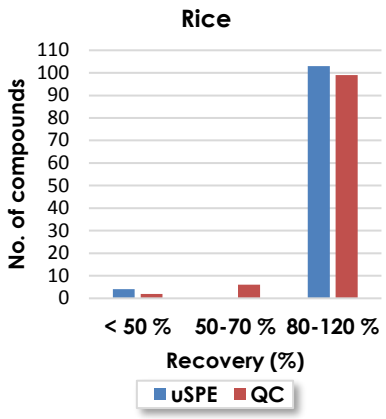


Figure 3. Recoveries of 106 compounds in the different matrices with µSPE (blue) and QC (dark red).

The most remarkable differences were observed in olive matrix: the average recovery for all compounds was 12 % higher in the µSPE approach, with more than half of the compounds in the range of 80-120 %. With the traditional clean-up step followed by solvent change, only 15 compounds were in this recovery range. Moreover, seven pesticide residues showed a recovery lower than 50 % with the QC method, namely o,p'-dicofol (40 %), endosulfan-alpha (49 %), fenvalerate (35 %), propiconazole (43 %), prothiofos (50 %), quinoxyfen (46 %) and tau-fluvalinate (49 %). The recovery of all these compounds was in the range of 50-70 % with the µSPE method.

In the case of orange, tomato, tea and rice, very similar results were obtained in terms of recovery for both methods, with the vast majority of compounds in the range of 80-120 %. However, in tea matrix, four compounds (azoxystrobin, buprofezin, fenazaquin, isocarbophos) could not be identified in the spiked sample at 10 µg/kg due to the 5-fold dilution of the extraction process, regardless of the clean-up and injection method followed (QC or µSPE).

The pesticide residue chlorothalonil is of especial interest because, although much less intense with the µSPE method (*see section 4.3*), this approach allowed to obtain recoveries close to 100 % in all matrices (with the exception of rice), whereas the traditional QC method with solvent change results in no recoveries (tomato and orange), or low recoveries (close to 60 %, olive and tea). The chromatograms in **Figure 4** show the recovery of chlorothalonil in orange (10 µg/kg) with both methods. This compound could be trapped in the dispersive clean-up salts, whereas it is more likely to be eluted from the µSPE cartridges.

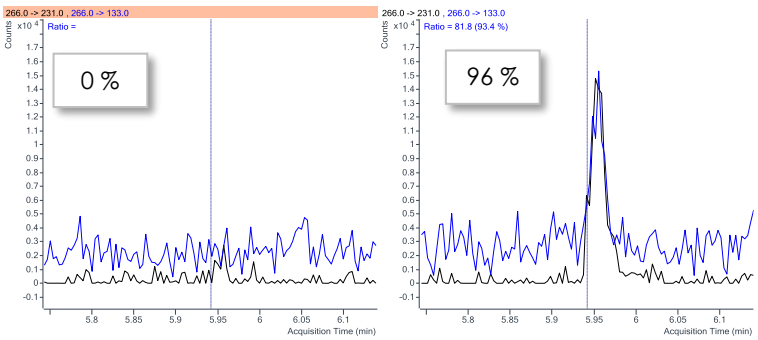


Figure 4. Recovery of chlorothalonil in orange with manual QC method (left) and µSPE method (right)

4.3 Signal intensity

The 2-fold dilution that entails the µSPE workflow followed in the present study resulted in lower chromatographic signals obtained for all compounds. On average, the signals provided by the 10 µg/kg standard were 2.6-2.9 times lower in the µSPE method than in the QC method. This effect was more intense in certain compounds for all matrices: cyprodinil and quinoxyfen (x5 times higher in the QC method) or dicofol (x3 to x9 times higher). Again, the compound chlorothalonil deserves especial emphasis, as its signals were 9 to 17 times more intense with the QC method (**Figure 5**, chlorothalonil); however, as discussed above, the traditional clean-up procedures followed by solvent change usually result in no recoveries of this compound.

Moreover, the loss of signal intensity resulted in certain compounds not being identified with the µSPE approach, as depicted in **Figure 5,** mepanipyrim. Therefore, following a µSPE approach, the minimum concentration that could be identified would be higher than 10 µg/kg in the following cases:

- Olive: fenazaquin, fenhexamid, mepanipyrim.
- Orange: endrin, fenazaquin, mepanipyrim, methidathion, pyraclostrobin, vinclozolin.
- Tomato: endrin, fenazaquin, mepanipyrm, prosulfocarb, pyraclostrobin.
- Tea: endrin, fenazaquin.
- Rice: fenazaquin, mepanipyrim, prosulfocarb.

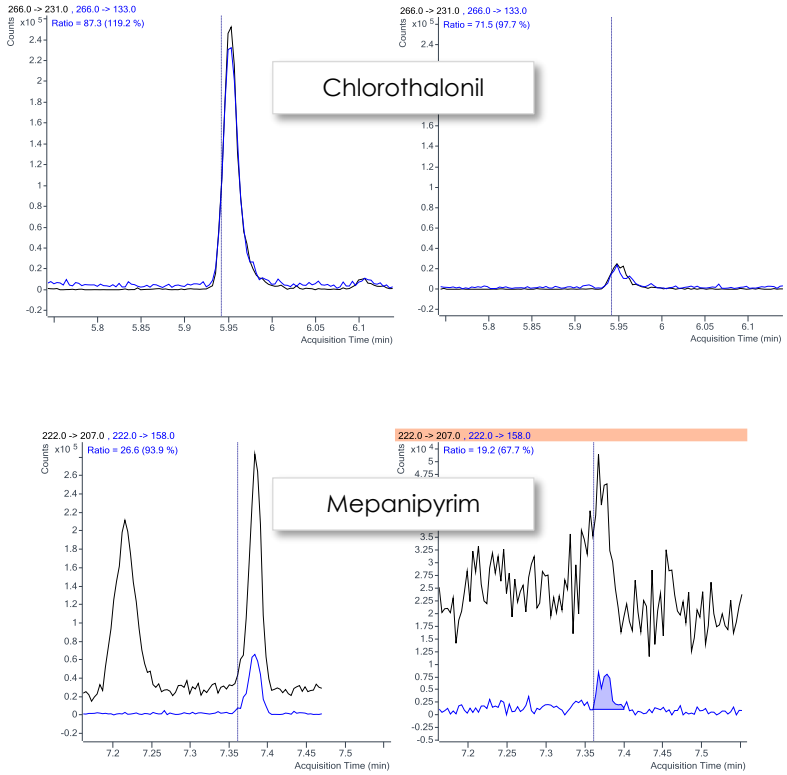


Figure 5. Chromatographic signal of chlorothalonil and mepanipyrim in olive (10 µg/kg standard) with QC method (left) and µSPE method (right). Scaled chromatograms

In tea matrix, although the average signal intensity was 2.3 times higher in the QC method, there was a significant amount of pesticide standards whose area was positively affected by the µSPE method. A total of nine compounds showed a more intense signal in the 10 µg/kg standard with the µSPE method and, in some cases such as buprofezin or pirimicarb, the signals provided by the QC method showed less than half the intensity of the ones from µSPE (even with the 2-fold dilution entailed in the latter method).

4.3.1. Avoiding the loss of sensitivity

As stated above, the µSPE method described in the present study entails a 2-fold dilution of the sample during the injection vial preparation, which results in lower chromatographic signals and, therefore, a loss of sensitivity. As a result, the areas of the vast majority of compounds were about 2.7 times lower than with the traditional QC method. In order to overcome this issue, a larger amount of sample can be injected -e.g. 2 µL instead of 1 µL. The use of solvent vent is in general advisable when injecting acetonitrile in gas chromatography and, when the injection volume rises to 2 µL, this injection mode is even more significant to protect the chromatographic columns from being damaged.

Orange µSPE extracts (2 µL) were injected using solvent vent injection mode to compare the chromatographic signals obtained with 1 µL injection volume. On average, the responses were 1.5 times higher than those corresponding to the injection of 1 µL, with no significant differences in recovery values or interferences. As regards the compounds which could not be initially identified in this matrix at 10 ppb, namely endrin, fenazaquin, mepanipyrim, methidathion, pyraclostrobin and vinclozolin, the increase in the sensitivity allowed in most cases to identify both transitions with a signal to noise ratio higher than 3.

However, the sample dilution is not a crucial step when working in routine analysis: the blank extracts can be evaporated and reconstituted with the same volume of the calibration solution. This would avoid the need of diluting both the samples and the calibration standards prior to the injection, so the loss of sensitivity would not take place. With this approach, however, the blank extract employed for the calibration curve would have been evaporated, conversely to the samples, so these matrices could be different. In the present study, the sample dilution was the preferred approach so as to ensure a correct assessment of the µSPE approach –i.e., comparability of all µSPE extracts. For routine analysis, it would be sufficient with an initial comparison of the evaporated and non-evaporated blank extracts to identify interferences in the non-evaporated extracts that could results in false positives. **Figure 6** shows the TICs obtained of the evaporated and non-evaporated blank orange extracts after the µSPE method; as can be seen, there are only minor differences, so the evaporated blank could be used for the quantitation of non-evaporated samples. The signals obtained with this matrix with the non-diluted µSPE method were very similar to those obtained with the manual QC methods, and no significant differences could be identified in terms of interferences and recoveries. The main drawback of this approach consists of the large volume of blank extract that is needed for the preparation of the calibration curve (300 µL per vial; a 5-point calibration curve plus a blank extract would require 1.8 mL of blank extract).

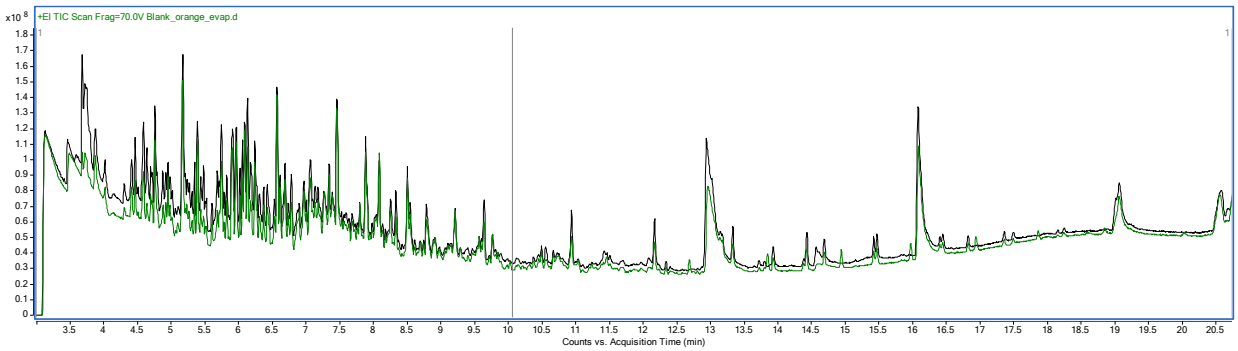


Figure 6. TIC chromatograms obtained with a GC-Q-TOF instrument for the µSPE blank orange extract: evaporated and reconstituted (green color) and non-evaporated (black color)

4.4 Interferences

The use of the automated µSPE method combined with no solvent change resulted in general in cleaner chromatograms with fewer matrix interferences, as discussed in *section 4.1*. This effect is more intense in complex matrices such as olive or tea: in the first case, seven compounds (diphenylamine, fenarimol, fenvalerate, isocarbophos, isofenphos-methyl, malathion-D10 and parathion) could not be identified at 10 µg/kg with the QC method due to high baselines and/or interferences. The same happened with isoprothiolane in tea matrix. Conversely, with the µSPE method, clear peaks were obtained for these compounds, without the need of employing specific clean-up sorbents (**Figure 7**).

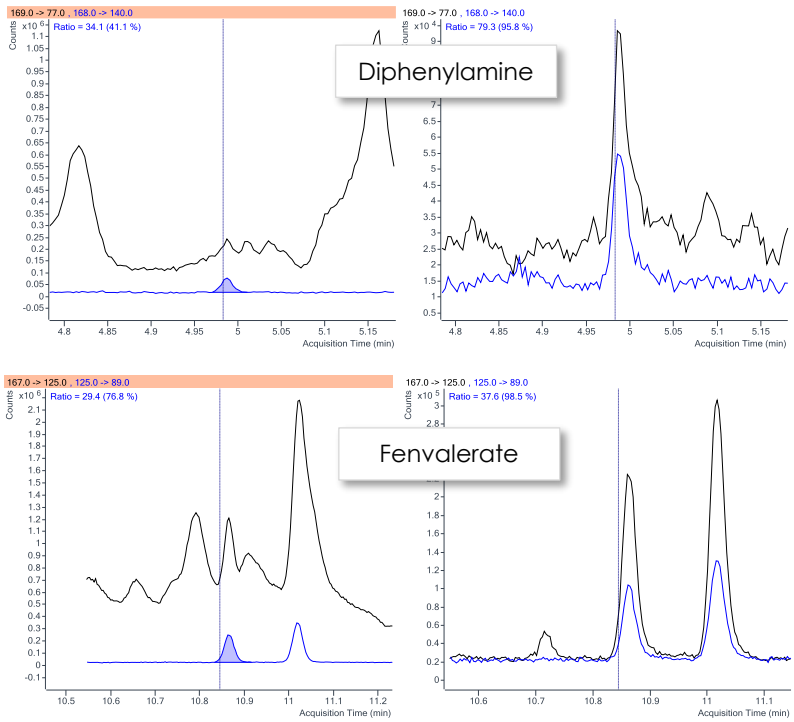


Figure 7. 10 µg/kg standard of diphenylamine and fenvalerate in olive with QC (left) and µSPE (right)

As tomato and rice are clean matrices with a reduced number of components, there were no interferences in the chromatograms of the pesticide residues and the use of µSPE did not result in significant differences. Similarly, in orange matrix, only a few compounds showed interferences and, in the majority of cases, they were still present with the use of the µSPE method.

5. Conclusions

The use of an automated µSPE clean-up workflow reduces significantly the laboratory work and allows to increase the sample throughput in routine analysis, as it 1) avoids the manual clean-up procedure, which is sometimes the longest step in any extraction method and 2) simplifies the vial preparation, as no solvent change is performed. Moreover, as only one clean-up is employed equally for all kind of samples, a higher homogeneity in the results can be obtained. The instrument maintenance could also be positively affected, as in general, cleaner extracts are obtained and, therefore, the lifespan of certain instrument parts (such as liner, columns or ion source) could be increased.

In general, the µSPE method results in recoveries which are very similar to those obtained with the manual clean-up approach, with some compounds being positively affected by the use of µSPE cartridges. For example, the use of the same solvent for the extraction and the analysis –i.e., with no evaporation step– makes it possible to obtain very good recoveries for the most volatile compounds such as dichlorvos, which can be partially lost during the solvent evaporation. Similarly, chlorothalonil, despite not being particularly volatile, showed much lower recoveries with the dispersive clean-up methods (as low as 0 % in some matrices) while reaching values of 100 % with the µSPE procedure. This method can be of particular interest with matrices that provide low recoveries of the analytes: in the case of olive, the vast majority of compounds underwent a 10 % increase in their recovery with the automated clean-up.

In the present work, the preparation of the calibration standards in the µSPE method involved the dilution of the blank extract with the pesticide mixture in solvent; for homogeneity reasons, all samples must be diluted to the same ratio (to ensure that the same amount of matrix will be injected and, therefore, there will be no matrix effects within the analysis). This sample dilution resulted in a loss of sensitivity that might affect the limits of quantification of the compounds. Some of the analytes (up to 6 %) seemed to be particularly affected by either the dilution or the use of cartridges, as they could not be identified at 10 µg/kg in most matrices (endrin, fenazaquin and mepanipyrim among others). However, in routine analysis, the sample dilution is not a necessary step, hence there are no losses of sensitivity issues expected with a µSPE method.

The µSPE cartridges release polymeric substances that can be observed in the first third of the TIC chromatograms and that might result in higher baselines when analyzing clean matrices. However, these compounds are not likely to result in interferences for the pesticide residues. Conversely, the baselines of complex matrices such as tea or orange are very positively affected by the use of these cartridges: most of the matrix interferences are retained and the baselines are significantly lower.

In conclusion, the use of an automated µSPE method is of particular interest when analyzing complex matrices or troublesome compounds, whose results might be highly improved with this technique. In the majority of cases, similar results are achieved by both techniques (manual or automated). However, the significant time and work savings that are achieved with the automatization of the clean-up step make this technique a very interesting and useful option for routine analyses that will simplify greatly the work of analytical laboratories over the next years.

6. References

[1] Lehotay, S. J., Han, L., Sapozhnikova, Y. Automated Mini-Column Solid-Phase Extraction Cleanup for High-Throughput Analysis of Chemical Contaminants in Foods by Low-Pressure Gas Chromatography—Tandem Mass Spectrometry, Chromatographia (79), 2016,1113–1130.

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