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Automated offline µ-SPE cleanup in GC-based multi-residue analysis: overcoming the challenges of fatty acids containing matrices

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Abstract

The aim of this work was to investigate and optimize the use of offline automated µ-SPE cleanup for the analysis of GC-amenable pesticides in fatty acids containing matrices, such as wheat and linseeds. The manual d-SPE and the automated µ-SPE procedure were compared in terms of analytical performance. Furthermore, different cartridge compositions, loading volumes and elution rates were tested and compared in order to find the best conditions for the cleanup of highly complex QuEChERS raw extracts. In µ-SPE, the key aspect was the PSA capacity for removal of fatty acids, and prevent overloading. The novelty of this research lies in the use of customized µ-SPE cartridges containing higher amounts of PSA, which proved to be the most effective strategy for minimizing matrix effects and enhancing method robustness.. Extracts obtained were cleaner compared to d-SPE, and the addition of analyte protectants (shikimic acid) was required to prevent adsorption of certain analytes in the GC system. The final µ-SPE workflow provided an efficient and reliable cleanup step the highly complex extracts rich in fatty acids tested and improved GC robustness reducing the need for GC-MS maintenance.

Keywords: Pesticides analysis**,** GC-MS/MS**,** Analytical Protectants**,** Clean-up, manual d-SPE, automated µ-SPE

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1.0 Introduction

The analysis of pesticide residues in food plays a crucial role for ensuring food safety. Pesticides are applied at different times, during growth, harvesting, transport and storage, to protect crops and food from pests, weeds, and diseases. An excessive use can lead to harmful residues for the public health. Hence, the importance of measuring pesticides at very low levels remains the ultimate goal for monitoring control programs. Gas-chromatography (GC) and liquid-chromatography (LC), mostly coupled with tandem mass spectrometry (MS/MS), are employed to accurately detect and quantify pesticide residues in food. Food is known to be one of the most complex samples to analyse, especially because of the presence of high concentration matrix constituents (e.g., fatty acids)[1]. The compounds naturally present in foods can interfere with the detection and quantification of pesticides in several ways, such as interfering with the signal (signal suppression or enhancement) resulting in the so called 'matrix-effect'[1,2]. Particularly in GC-based methods, co-extractants can also negatively influence the performance of the instrument. Two main options to reduce adverse effects of the matrix are dilution and clean-up of the final extract prior the analysis. Diluting the sample with clean solvent is often used in LC-based analysis while clean-up strategies based on matrix-sorbent material interaction are typically carried out prior GC analysis. The main drawback of dilution is the reduction of the in-vial concentration which for certain analytes may result in limits of quantification (LOQs) not fit for maximum residue limits (MRLs) compliance testing or monitoring for risk assessment. Interaction between sorbent material and some target pesticide is a concern when clean-up strategies are applied for reducing adverse effects of co-extracted matrix (e.g., interaction between planar pesticides and graphitized carbon black (GCB)[3]). Overloading the SPE sorbent with matrix may be an issue with certain food commodities (e.g. fatty acids to be absorbed on amino-based sorbents like PSA). For these main reasons both approaches must be well investigated and optimized before being introduced into the final analytical protocol.

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Sorbent materials can be used in dispersive mode (d-SPE) or using conventional SPE cartridges. d-SPE is quick, while SPE cartridges can be more effective but involve a more laborious manual procedure.

The first appearance of µ-SPE, dating back to 2015 and initially referred to as automated cartridge-SPE (c-SPE), for extracts' cleanup was reported by Morris & Schriner [4], who used the Instrument Top Sample Preparation (ITSP) mini-cartridges based on zirconia sorbent for fatty acids and pigments removal from avocado, citrus, and buttercup squash, improving extract cleanliness, and enhanced reproducibility and throughput for LC-MS/MS pesticide residue analysis of 263 analytes of interest.

The year after, Lehotay et al. introduced automated mini-SPE cleanup combined with low-pressure GC-MS/MS, enabling high-throughput analysis of pesticides and environmental contaminants in foods with reduced manual labour, improved reproducibility, and minimized matrix effects [5]. The method was validated for the analysis of 54 pesticides and 43 environmental contaminants in 10 different food matrices.

Sapozhnikova et al. reported for the first time the online combination of mini-SPE cleanup coupled to low-pressure GC-MS/MS for several pesticides and environmental contaminants in cattle, swine and poultry muscle tissues [6] and, later, in catfish [7].

Other than pesticides, automated mini-SPE was employed for the cleaning of extracts in order to improve the detection of warfare agents in the environment (water and soil) and polycyclic aromatic hydrocarbons in food oils [8,9].

Several scientific manuscripts related to mini-SPE were published in 2021. Hakme and Poulsen evaluate the use of mini-SPE for the clean-up of the analysis of pesticides in cereals demonstrating high efficiency in removing matrix components, reaching lower LOQs for several pesticides [10]. Goon et al., developed an automated mini-SPE cleanup method for pesticide residue analysis in various spice matrices, including chili powder, turmeric, black pepper, cumin, coriander, and cardamom [11]. Noteworthy, Lehotay et al., combined mini-SPE clean-up with a modification of the QuEChERS

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sample preparation method, called "QuEChERSER", for the combined detection of pesticides, veterinary drugs, and environmental contaminants in beef, catfish, and animal derived matrices [12–15]. In the same period, QuEChERSER was again coupled to mini-SPE for the analysis of pesticide residues in hemp and hemp products [16].

A new design of cartridges, defined as septumless μ-SPE mini-cartridge, was described for the first time in 2022 by Michling & Lehotay [17]. This type of cartridges are characterized by the absence of a septum reducing the chance of leakage and of syringe needle break, although more susceptible to moisture uptake by $MgSO_4$. A different sealing mechanism allows the usage of higher flow rates (> 10 μL sec^{-1} compared to the ≤ 2 μL sec^{-1} for the ITSP cartridges). In addition, the septumless μ-SPE mini-cartridges offer the possibility of a larger sorbent bed, whereas ITSP cartridges are limited to 45 mg. The authors demonstrated that the septumless μ-SPE mini-cartridges enabled a faster, reliable, and highly reproducible automated cleanup, achieving excellent recoveries and low relative standard deviations (RSDs) for over 250 analytes across diverse food matrices, making them a robust solution for high-throughput QuEChERSER analyses. These new μ-SPE cartridges have been used in different applications in the recent past as clean-up strategy prior the analysis of pesticide residues, natural toxins and contaminants [18–22]. Recently, a different use of μ-SPE cartridges in combination with a centrifuge, defined as centrifugal μ-SPE, was reported by Michling and Lehotay, allowing this approach to be used without requiring the complete PAL system although with some limitations [23]. Compared to d-SPE, the volume needed for the μ-SPE cleanup is smaller (≤ 500 μL) which makes it ideal for miniaturization of QuEChERS sample preparation. Several scientific publications have proven that scaling down is possible without compromising the representativeness of the analytical portion extracted, even without the use of cryo-milling [24–26].

The combination of mini-QuEChERS and μ-SPE can indeed prove to be a highly advantageous approach in terms of environmental impact, cost reduction, and reduced analyst exposure to toxic substances.

The aim of this work is to investigate and optimize the use of offline automated µ-SPE cleanup for the analysis of GC-amenable pesticides in fatty acids containing matrices, such as wheat and linseeds. According to the SANTE guidelines[27], wheat and linseeds can be considered representative commodities of Group 5 ("High starch and/or protein content and low water and fat content") and Group 3 ("High oil/fat content and very low water content"), respectively. The manual d-SPE and the automated µ-SPE procedure were compared in terms of analytical performance. Furthermore, different cartridge compositions, sorbent amounts, loading volumes and elution rates were tested and compared in order to find the best conditions for the cleanup of highly complex QuEChERS raw extracts.

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2.0 Experimental section

2.1 Chemicals and Standards

Pesticide standards were purchased from LGC Standards (Wesel, Germany) and Sigma Aldrich Chemie B.V. (Zwijndrecht, The Netherlands). The extraction solvent ACN (LC-MS grade) was purchased from Biosolve (Valkenswaard, the Netherlands). Acetic acid, ammonium formate, magnesium sulfate ($MgSO_4$), sodium acetate anhydrous (NaOAc), and Shikimic acid were obtained from Sigma Aldrich Chemie B.V. (Zwijndrecht, The Netherlands). Water was purified using a Milli-Q system (Millipore, Burlington, MA, USA). An Acetate Mix conforming to AOAC 2007.01, consisting of 4 g of $MgSO_4$ and 1 g of NaOAc, was obtained from HPC Standards GmbH (Cunnersdorf, Germany). The d-SPE tubes (containing 150 mg of $MgSO_4$, 25 mg of C18 and 25 mg of PSA) and Bondesil-PSA 40µm were obtained from Agilent (Santa Clara, CA, USA). One type of commercialized µ-SPE cartridges, namely GCQuE1-45,was purchased from CTC analytics (Zwingen, Switzerland). Three different types of custom-made µ-SPE cartridges were kindly provided by CTC analytics and contained higher amounts of PSA. The composition of the different µ-SPE cartridges is described in Table S1.

2.2 Sample

Wheat flour and linseed samples were purchased in a local organic shop. Wheat flour was not subjected to any pre-treatment. Linseed was homogenized by a conventional milling procedure at ambient temperature. Linseed was stored at − 20 °C , whereas wheat was kept at ambient temperature until use.

2.3 Sample preparation

Sample preparation was carried out using the acetate-buffered QuEChERS protocol [28]. Briefly, 2.50 ± 0.05 g of each sample was weighted in a 50-mL centrifuge tube, followed by the addition of 7.5

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mL of distilled water, and 30 s of hand shaking. Next, 10 mL of ACN (1% AA) was pipetted into the tubes followed by agitation head-over-head for 30 min. A mixture of salt containing magnesium sulphate (4 g) and sodium acetate (1 g) was added, and the tube was shaken for 5 min to induce salting-out and phase separation. The samples were centrifuged at 3500 r.p.m. for 5 min at 10 °C. After the extraction two different clean-ups were performed as follow:

d-SPE: for this the Agilent d-SPE tubes were used containing 150 mg $MgSO_4$, 25 mg C18 and 25 mg PSA, to which an additional 225 mg of PSA was added. One mL of raw extract was added to the tube together with 25 µL of PCB-198 (1 µg mL^{-1}), followed by thoroughly shaking and centrifuge for 5 min at 13,000 r.p.m. Subsequently, 10 µL of ACN (1% AA) or standard mix (at different concentration) were added to 100 µL of the supernatant for the real samples or for the matrix matched standards, respectively.

µ-SPE: for the final µ-SPE clean-up 300 µL of extract (from a vial containing 1 mL raw extract + 25 µL of 1 µg mL^{-1} of PCB-198) was cleaned at a speed of 5 µL sec^{-1}. Afterwards, 90 µL of cleaned extract were transferred to the final vial to which 5 µL of ACN (1% AA) or standard mix (at different concentration) were added for the real samples or for the matrix matched standards, respectively. Finally, 5 µL of Shikimic acid at a concentration of 4 mg mL^{-1} (1 µg on column) were added to the vial followed by a final mixing step. All the steps are illustrated in Figure 1. The final extract (0.25 g mL^{-1}) is then ready for analysis.

Please insert Figure 1

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2.4 Instrumentation

2.4.1 GC-MS/MS

All the GC-MS/MS experiments were carried out using a GC 7890 B and a G7013 B triple quadrupole MS (Agilent Technologies, Santa Clara, CA, USA). Data were acquired and processed by the use of the MassHunter software (v. B.09.00). The GC was equipped with the 7693 autosampler and the Multi-Mode Injector (MMI). A ultra inert splitless liner (Agilent 5190-4006) was installed in the injector port. Separation was performed on a Rtx-CLPesticides 30 m × 0.25 mm ID × 0.25 µm d_f (Restek nr. 11123) column. Helium was used as carrier gas with a flow of 1 mL min^{-1}. The injection volume was 5 µL and injection was performed in solvent vent mode at 75 mL min^{-1} for 0.065 min followed by split 50 mL min^{-1} after 4 min. The temperature of the injector was as follows: 60 °C (0.3 min) with 900 °C min^{-1} to 285 °C (1.5 min), with 900 °C min^{-1} to 350 °C (15 min.). Temperature oven program: 60 °C (2 min) to 150 °C at 20 °C min^{-1}, to 280 °C at 10 °C min^{-1}, to 320 °C (5 min) at 25 °C min^{-1}, total run time 26.1 min.

Mass spectrometry conditions: the temperature of the interface was 300 °C; the ion source temperature was 250 °C, with electron ionization at 70 eV. Nitrogen was used as collision gas with a collision flow of 1.5 mL min^{-1} and a quadrupole temperature of 150 °C. Two multi reaction monitoring transitions were measured for each pesticide. A list of all the transitions can be found in Table S2.

2.4.2 GC-Q-Orbitrap

All the GC-Q-Orbitrap experiments were carried out on a Q-Exactive system (Thermo Fisher Scientific, Bremen, Germany) consisting of a TRACE 1310 GC and a hybrid Q-Orbitrap mass spectrometer. Data were acquired and processed using TraceFinder software (v. 4.1). The GC was equipped with a TriPlus RSH™ autosampler and a programmed temperature vaporizer injector (PTV). A ultra inert liner, (Restek RT-21117-216) was installed in the injector port. Separation was

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performed on a Rxi-5-SILMS 30 m × 0.25 mm ID × 0.25 µm d_f (Restek nr. 13623) column. Helium was used as carrier gas with a flow of 1.2 mL min^{-1}. Injection volume was 3 µL and it was performed in solvent vent mode at 50 mL min^{-1} for 0.10 min followed by split 50 mL min^{-1} after 1.2 min. The temperature of the injector was as follows: 70 °C (0.1 min) with 300 °C min^{-1} to 300 °C (16.5 min). Temperature program: 80 °C (1 min) to 185 °C at 30 °C min^{-1}, to 280 °C (1 min) at 10 °C min^{-1}, to 320 °C (5.86 min) at 35 °C min^{-1}, total run time 21.5 min.

Mass spectrometry conditions: the temperature of the interface was 300 °C; the ion source temperature was 290 °C, with electron ionization at 70 eV.

Full Scan (FS) acquisition was performed in profile mode using a *m/z* range of 75–500. The resolving power was set at 60,000 Full Width Half Maximum (FWHM) at *m/z* 200. The automatic gain control (AGC) target was set at 3e6 ions, with the maximum ion injection time set to auto. Tuning and mass calibration were carried out before each sequence. Internal mass calibration was automatically performed by the instrument using three background ions from the column bleed as lock mass ($C_5H_{15}O_3Si_3^+$, 207.03235; $C_7H_{21}O_4Si_4^+$, 281.05114; and $C_9H_{27}O_5Si_5^+$, 355.06994). TraceFinder 4.1 was used to process the data and exact masses of two ions (quantifier and qualifier-1) for each compound were selected. The list of analytes and their exact masses of the ions are provided in Table S3.

2.5 Methods:

2.5.1 Initial clean-up comparison: d-SPE vs GCQuE1-45 µ-SPE cartridges

In order to compare the robustness of the GC-MS/MS system, the injection of the same QuEChERS wheat extract, spiked at 12.5 µg kg^{-1} (corresponding to 50 µg kg^{-1} in matrix), cleaned via d-SPE and µ-SPE was evaluated. Briefly, for d-SPE, multiple aliquots of 1 mL of wheat raw extract were added to a commercial d-SPE Eppendorf tubes containing 150 mg of $MgSO_4$, 25 mg of C18, and 25 mg of PSA to which an extra 225 mg of PSA was added. A volume of 25 µL of PCB-198 (1 µg mL^{-1}) was

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added to the tube as injection internal standard, followed by thoroughly shaking and centrifuge for 5 min at 13000 r.p.m. Subsequently, 990 µL was collected from the d-SPE tube and 10 µL of ACN (1% AA) was added to the final vial. The cleaned extracts were merged together and then split in 23 vials and injected subsequently in the GC-MS/MS system.

The µ-SPE clean-up was performed using the GCQuE1-45 cartridge containing 20 mg of $MgSO_4$, 12 mg of C18, 12 mg of PSA, and 1 mg of GCB. Briefly, 1 mL of extract + 25 µL of 1 µg mL^{-1} of PCB-198 was added individually to 20 vials and, from each vial 300 µL of extract was cleaned at a speed of 5 µL sec^{-1}. Afterwards, 95 µL of cleaned extract were transferred to the final vial together with 5 µL of ACN (1% AA) for injection into the GC-MS/MS system.

Samples were injected on two different days on the GC-MS/MS system, each time using a new liner and a freshly trimmed front section of the column. Before injecting the spiked samples, the liner was primed with 5 consecutive injection of raw wheat extract.

Besides the robustness comparison described above, the removal of matrix co-extractants was also gravimetrically assessed. For this, after QuEChERS extraction, 2 mL of raw uncleaned extract, 2 mL of d-SPE cleaned, and 2 mL of µ-SPE cleaned were evaporated using a TurboVap. The difference in vial weight before and after evaporation was calculated in order to obtain the solid residue expressed as mg mL^{-1}. For the d-SPE experiment, the sorbent composition used to clean 9 mL of extract consisted of 600 mg $MgSO_4$, 360 mg C18, 360 mg PSA, and 30 mg GCB. This composition was selected to maintain the same sorbent-to-extract ratio as in the µ-SPE procedure, enabling a direct comparison of the clean-up efficiency between the two strategies.

2.5.2 Custom made and commercial µ-SPE cartridges comparison

Three different types of custom-made cartridges together with one of the commercially available versions were compared. The composition of all cartridges is described in Table S1. Briefly, the

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commercially available cartridges contain 12 mg of PSA, while the custom-made ones contain PSA in increasing amounts, 24, 36 and 48 mg.

In order to compare the ability of the different cartridges to remove co-extractants and especially free fatty acids, wheat and linseeds were extracted and then cleaned using the four different cartridges. As for the previous experiment, 1 mL of raw extracts spiked with 12.5 µg kg^{-1} (corresponding to 50 µg kg^{-1} in matrix) of the pesticides mix + 25 µL of PCB-198 (1 µg mL^{-1}) were added to the vials and 300 µL of extracts was cleaned at a speed of 5 µL sec^{-1}. The analysis was carried out using a GC-Q-Orbitrap. The total ion current (TIC) was then compared, with particular attention paid to the percentage of fatty acids removed. The cleanup recovery of the pesticides was used to compare the influence of the different cleanup cartridges. For this experiment, shikimic acid was not employed as an analyte protectant. Additional experiments involving dilution with a tomato extract and the use of shikimic acid to improve the signal of selected pyrethroids have been included in the corresponding section of the Results and Discussion (section 3.2. Custom made and commercial µ-SPE cartridges comparison).

2.5.3 d-SPE vs CM-02 GCQuE µ-SPE. Clean-up comparison

As for the experiment described in Section 2.5.1, to compare the robustness of the GC-MS/MS system, the injection of the same QuEChERS wheat extract, spiked at 12.5 µg kg^{-1} (corresponding to 50 µg kg^{-1} in matrix), cleaned via d-SPE and µ-SPE was evaluated. The d-SPE clean-up was already described in section 2.5.1. Regarding the µ-SPE clean-up, it was performed using the CM-02 GCQuE µ-SPE, consisting of 20 mg of $MgSO_4$, 12 mg of C18 and **36** mg of PSA. Briefly, 1 mL of extract + 25 µL of 1 µg mL^{-1} of PCB-198 was added individually to 20 vials and, from each vial 300 µL of extract was cleaned at a speed of 5 µL sec^{-1}. Afterwards, 90 µL of cleaned extract is moved to the final vial were 5 µL of ACN (1% AA) and 5 µL of Shikimic acid at a concentration of 4 mg mL^{-1} (1 µg on column) was added to the vial and ready to be injected in the GC-MS/MS system.

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Samples were injected on two different days on a GC-MS/MS system with a new liner and a freshly trimmed front section of the column. Before injecting the spiked samples, the liner was primed with 5 consecutive injection of raw wheat extract.

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3.0 Results and Discussion

3.1. Initial clean-up comparison: d-SPE vs GCQuE1-45 µ-SPE cartridges

As a first step, the in-house d-SPE method was compared with the commercially available GCQuE1-45 cartridges (see Section 2.5.1 for the method details). The aim was to assess whether, despite the relative small amount of PSA, the conventional µ-SPE cartridges could already be sufficient to obtain an extract clean enough to not compromise the GC-MS/MS performance in a typical overnight sequence. To this extend, the robustness of the GC system was first evaluated by injecting the same wheat extract cleaned by d-SPE and µ-SPE on two different days respectively, using a new liner and a freshly trimmed front section of the column each time. In both experiments the standard deviation was calculated for the response factor of each pesticide based on 23 injections.

As illustrated in Figure 2, the results obtained from the robustness test using the d-SPE method (blue bars) can be considered satisfactory (with exception of dichlorvos). Conversely, µ-SPE using the commercially available GCQuE1-45 cartridges showed poorer results (orange bars), with RSDs exceeding 20% for 12 pesticides, most notably for p,p'-DDE, α -endosulfan, p,p'-DDT, and methoxychlor. To demonstrate that these results are related to a deterioration in instrumental performance, the same extract was diluted sixfold after µ-SPE and re-injected on a different day. The results (yellow bars) show that, after dilution, the majority of pesticides exhibit RSD values below 20%, demonstrating that the system remained stable throughout the entire 23 injection batch.

Please insert Figure 2

As further evidence of the inability of the conventional cartridges to efficiently remove matrix components, the solid residue was determined for the raw extract and for the extracts obtained after

clean-up by d-SPE and µ-SPE. Moreover, different clean-up flow rates were also evaluated in order to assess whether this parameter could influence the effectiveness of the µ-SPE clean-up and an extra d-SPE tube with the same composition (in terms of sorbent-to-extract ratio) as µ-SPE was included. The experimental details are described in Section 2.5.1.

Table 1 reports the results related to the solid residual obtained after the different experiments. While the in-house d-SPE method efficiently removed matrix (93%), results were clearly worse for µ-SPE (55%), thus confirming that a larger amount of matrix-derived material is introduced into the instrument, accelerating instrumental deterioration. Nevertheless, as shown in Table 1, when the same sorbent-to-extract ratio is used (d-SPE same ratio as µ-SPE), µ-SPE exhibits a slightly higher removal efficiency compared to d-SPE. The different clean-up flow rates do not play a crucial role, giving similar results in terms of matrix removal.

Please insert Table 1

The extracts were also injected on the GC-Q-orbitrap and the TIC was compared in order to evaluate the removing of the GC-amenable co-extractants for the different cleanup options and without any cleanup. As illustrated in Figure S1, while d-SPE (red TIC) allowed efficient removal of fatty acids (between 9 to 11 min), these were clearly visible in the extracts cleaned by µ-SPE, regardless of the clean-up flow rate applied.

In conclusion, it became clear that the commercially available cartridges did not sufficiently remove co-extractants and, consequently, did not enable the GC–MS/MS system to maintain its performance over the duration of a conventional analytical batch. The presence of the fatty acid peaks indicated that these were insufficiently removed, attributed to overloading of the µ-SPE / insufficient amount

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of PSA in the cartridge. To address this issue, potential custom-made cartridges characterized by a higher amount of PSA were investigated in order to enhance fatty acid removal. Alternative approaches to address the issue related to the matrix removal, based on the use of commercial µ-SPE cartridges, include dilution of the crude extract prior to clean-up or the cleaning of volumes lower than 300 µL. However, extract dilution may negatively impact the method LOQs. Cleaning a volume substantially lower than 300 µL caused issues with a low eluent fraction / incomplete elution due to the dead volume of the sorbent material (approximately 50 µL).

3.2. Custom made and commercial µ-SPE cartridges comparison

In order to improve fatty acid removal, four different types of cartridges (one commercially available and three custom-made) were used to perform the clean-up of wheat and linseed QuEChERS raw extracts. All these cartridges, as described in detail in Section 2.5.2, are characterized by increasing amounts of PSA.

The PSA removes fatty acids by binding their carboxylic acid groups via acid-base interactions and hydrogen bonding, retaining them on the sorbent while the target pesticides remain in solution[29].

Figures 3 and Figure S2 show a comparison of the normalized TICs for all the different types of cartridges used for the cleanup of wheat and linseed extracts, respectively. As is clear from both figures, increasing the amount of PSA drastically improves the removal of fatty acids, which elute approximately in the middle of the chromatogram (fatty acid zone in Figure 3). In contrast, for the commercially available cartridges, it is evident that overloading occurs.

Please insert Figure 3

In addition to evaluating matrix removal capability, pesticide recovery after cleanup was also assessed. To this extend, the pesticide mixture was added to the extract before and after the cleanup step, in order to compare the response factors obtained for each individual pesticide.

An overview of the cleanup recoveries obtained for the two different matrices used in this study are reported in Table 2, while the cleanup recoveries obtained for the individual pesticides are reported in Table S4. Briefly, the majority of pesticides exhibited recoveries higher than 70%. This demonstrates that none of the different cartridge types employed caused significant retention, hence losses of any analyte of interest.

Please insert Table 2

Notably, for the custom 02 and custom 03 cartridges (containing the highest amounts of PSA), no signal was obtained for some pesticides. This included pyrethroids such as cypermethrin. It is well known that cypermethrins and many other pyrethroids tend to be adsorbed by active sites in the liner. Presence of matrix components in the extracts reduces this effect by masking the active sites of the glass and provides better recoveries of these pesticides[30]. Given the excellent clean-up performance of cartridges 02 and 03, and considering that the recoveries of these compounds were optimal for all the other cartridges, it was hypothesized that the high clean-up efficiency caused these compounds to be adsorbed by the glass liner. In order to confirm this hypothesis, the extract after clean-up was injected as such and after a 1:1 dilution with a tomato extract.

As shown in Figure S3, depending on whether the linseed sample was diluted with a tomato extract (green box) or injected undiluted (red box), the peaks corresponding to cypermethrin were present or absent, respectively. This behaviour was observed both when fortification was performed before the

clean-up (blue rectangle) and when it was carried out after the clean-up (yellow rectangle), in order to exclude any losses of cypermethrin by the cartridges. In addition, it can be noted that the peak areas (and the response factors) corresponding to the extract diluted 1:1 with the tomato extract were consistent, regardless of whether fortification occurred before or after the clean-up, proving that clean-up had not a negative impact in the clean-up recovery of this set of four compounds.

So, on one hand, clean extracts are beneficial to avoid issues related to instrumental stability and maintenance. On the other hand, (some) matrix is needed to shield active sites to ensure proper response and peak shape. In order to obtain both advantages, as demonstrated, it is possible to dilute the extract after clean-up with a not complex matrix extract (such as a tomato) or to rely on the use of compounds able to shielding the active sites in the system. Analytical protectants (APs) are compounds intentionally added to the vial to prevent analyte loss and signal instability caused by interactions with active sites in the inlet, liner, column, or detector [31,32]. As demonstrated by Rodríguez-Ramos et al., 1 µg of Shikimic acid on column enabled the achievement of optimal results for multi-pesticide methods and organic contaminants. [33].

Although the use of a tomato extract can be considered sufficient to prevent liner adsorption of susceptible analytes, the use of APs might be preferable. This is because their use can be controlled and they provide consistent and reproducible results. Contrarily, the use of a simple matrix extract requires the availability of a blank and may vary depending on the vegetable variety and the time of year. For this reason, the use of shikimic acid at a concentration of 1 µg on-column was tested in order to prevent the adsorption/degradation issues with more polar and labile pesticides in the GC inlet and column. To demonstrate the effectiveness of shikimic acid, Figure S4 shows the four cypermethrin peaks obtained from a wheat sample spiked at 12.5 ng mL^{-1} and injected during a batch of 20 analyses. As can be observed, the peak areas remained stable throughout the sequence, with RSDs of 5.9 %, for cypermethrin I.Finally, the custom 02 cartridges, were considered as the best

candidate to be used in all subsequent experiments, as it effectively removed fatty acids and no improvement was observed for custom 03 with an even higher amount of PSA.

3.3. d-SPE vs CM-02 GCQuE µ-SPE. Clean-up comparison

In order to confirm that these cartridges allow optimal matrix removal in terms of instrumental performance, the same experiment described in Section 3.1 was repeated. As illustrated in Figure 4 the grey lines show the RSD% results obtained when CM-02 GCQuE µ-SPE cartridges were used for extract clean-up, in comparison with the blue lines corresponding to the in-house d-SPE method.

Please insert Figure 4

As is evident, particularly when compared with the commercial µ-SPE cartridges described in Figure 1, the RSD% values for the majority of pesticides, in a sequence of 23 injections, are now optimal. In comparison with the d-SPE method, most pesticides also show an improvement in RSD%. This demonstrates that these cartridges provide improved matrix removal, resulting in more robust and repeatable instrumental performance.

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4.0 Conclusions

In this study, a comparison between an in-house sample clean-up method based on d-SPE and an automated µ-SPE approach was performed.

The results clearly showed that the presence of additional PSA enabled efficient removal of fatty acids, thereby drastically improving system stability. Moreover, the use of shikimic acid, as an analyte protectant, proved to be an effective solution to obtain consistent response of pesticides prone to adsorption in the inlet in case of well cleaned extracts. The clean-up recoveries of the targeted pesticides were evaluated, with the majority exceeding 70%, thereby indicating that no significant retention occurred. Overall, the µ-SPE workflow herein reported represent a promising cleanup approach for highly complex extracts rich in fatty acids showing the potential to enhance GC robustness and contribute to reduced GC-MS maintenance needs.

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Author contributions

Ivan Aloisi: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review and editing. **Lisa Elsinga**: Formal analysis, Methodology, Validation, Writing – review and editing. **Michel Willemsen:** Formal analysis, Methodology, Validation, Writing – review and editing. **Hans Mol:** Funding acquisition, Project administration, Conceptualization, Resources, Writing – review and editing.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data is reported in the article and provided in the supporting information files.

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Figure legends

Figure 1. Final µ-SPE workflow optimized for the clean-up of QuEChERS extracts.

Figure 2. Comparison of the standard deviation (RSD%) for the 69 pesticides after 23 consecutive injection of samples cleaned vial d-SPE (blue), µ-SPE (orange), and µ-SPE followed by 6 times dilution (yellow).

Figure 3. Comparison of the normalized TICs for all the different types of µ-SPE cartridges used for the cleanup of wheat extract.

Figure 4. Comparison of the standard deviation (RSD%) for the 69 pesticides after 23 consecutive injection of samples cleaned vial d-SPE (blue) and µ-SPE with extra PSA (grey).

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Table 1. Wheat solid residual and % of matrix component removal

	Residue $mgml^{-1}$	% of removal
RAW	2.8	
d-SPE	0.2	93
d-SPE same ratio as µ-SPE	1.7	39
µ-SPE (5µL sec^{-1})	1.3	53
µ-SPE (3µL sec^{-1})	1.6	44
µ-SPE (1µL sec^{-1})	1.3	54

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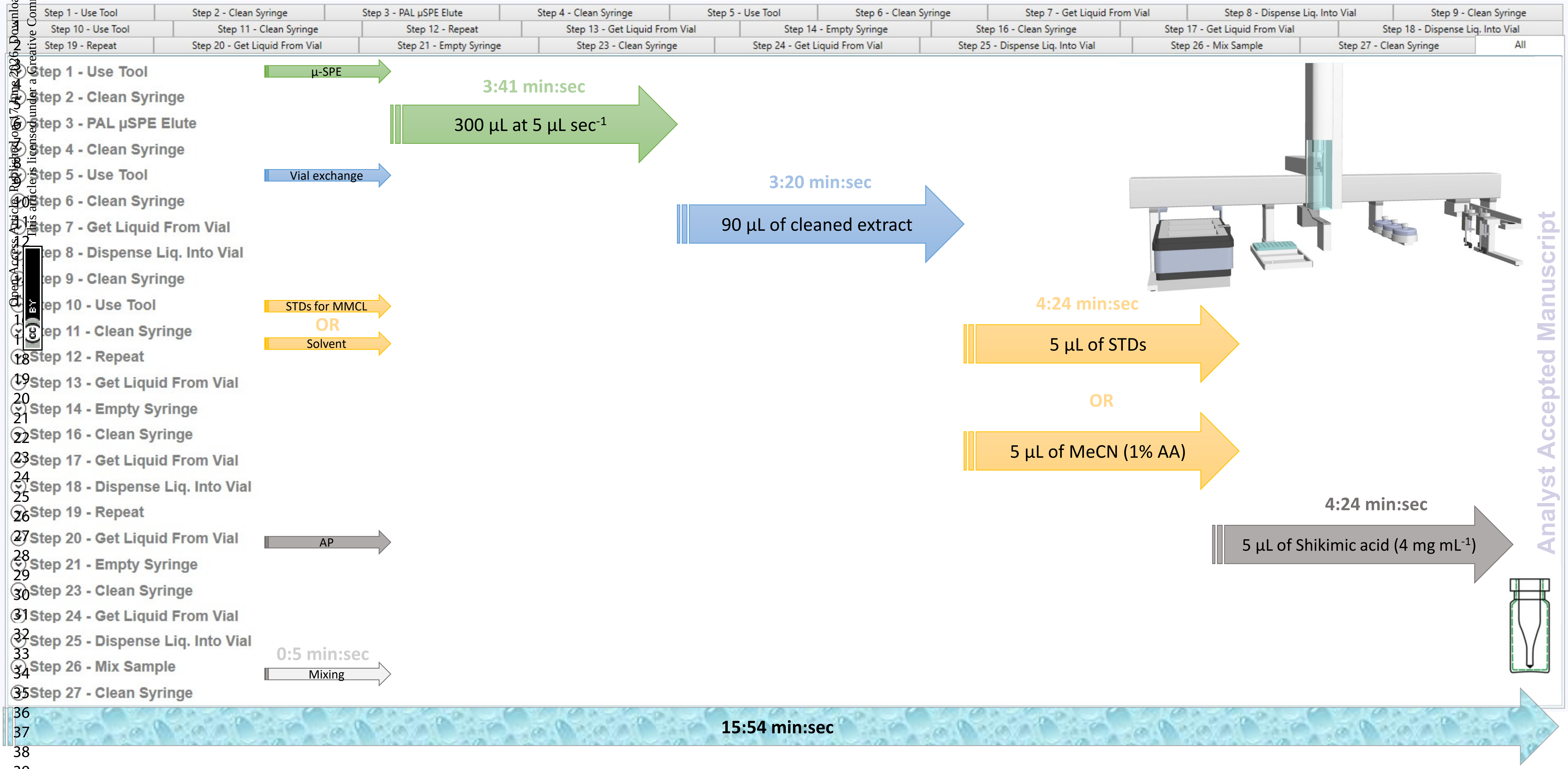
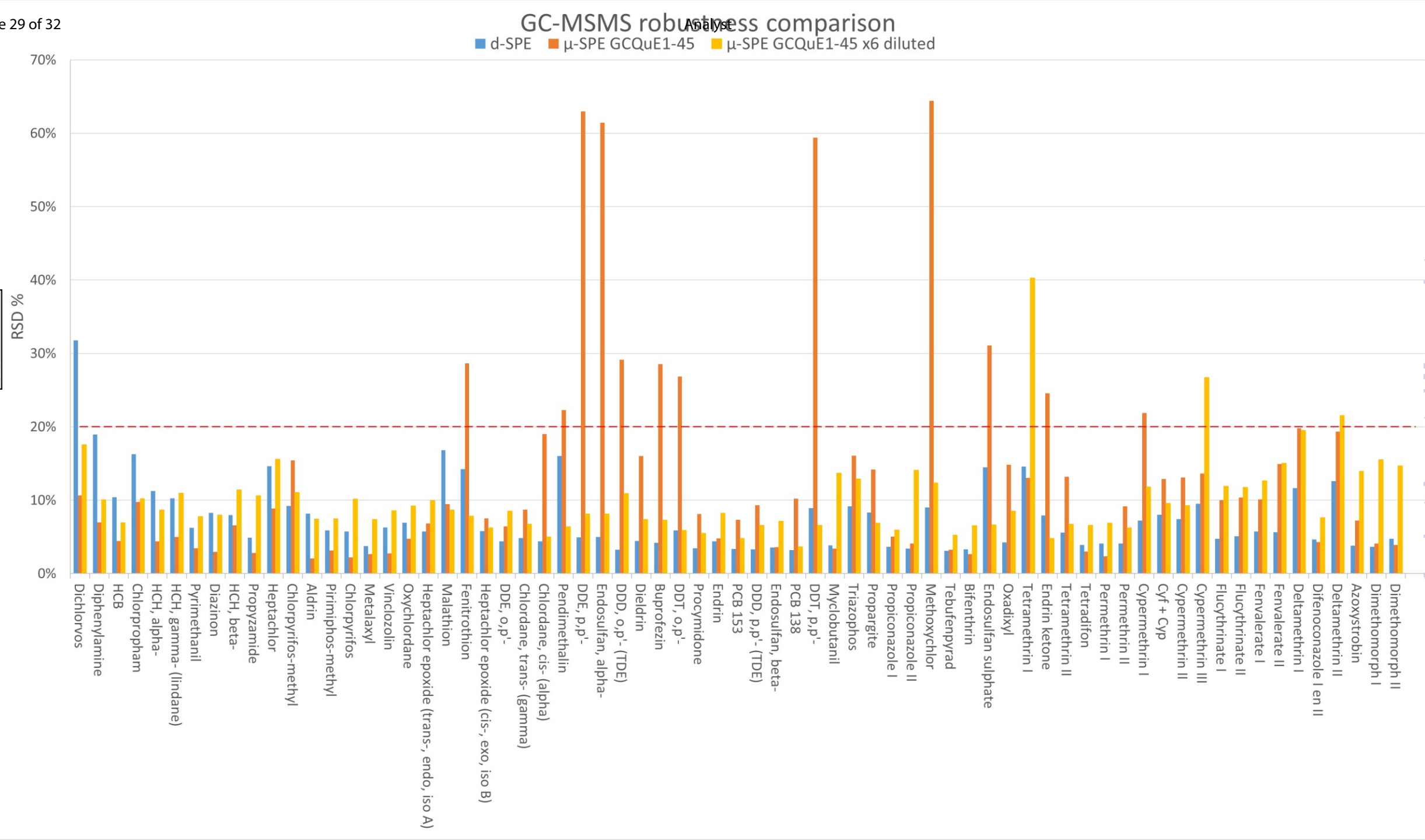
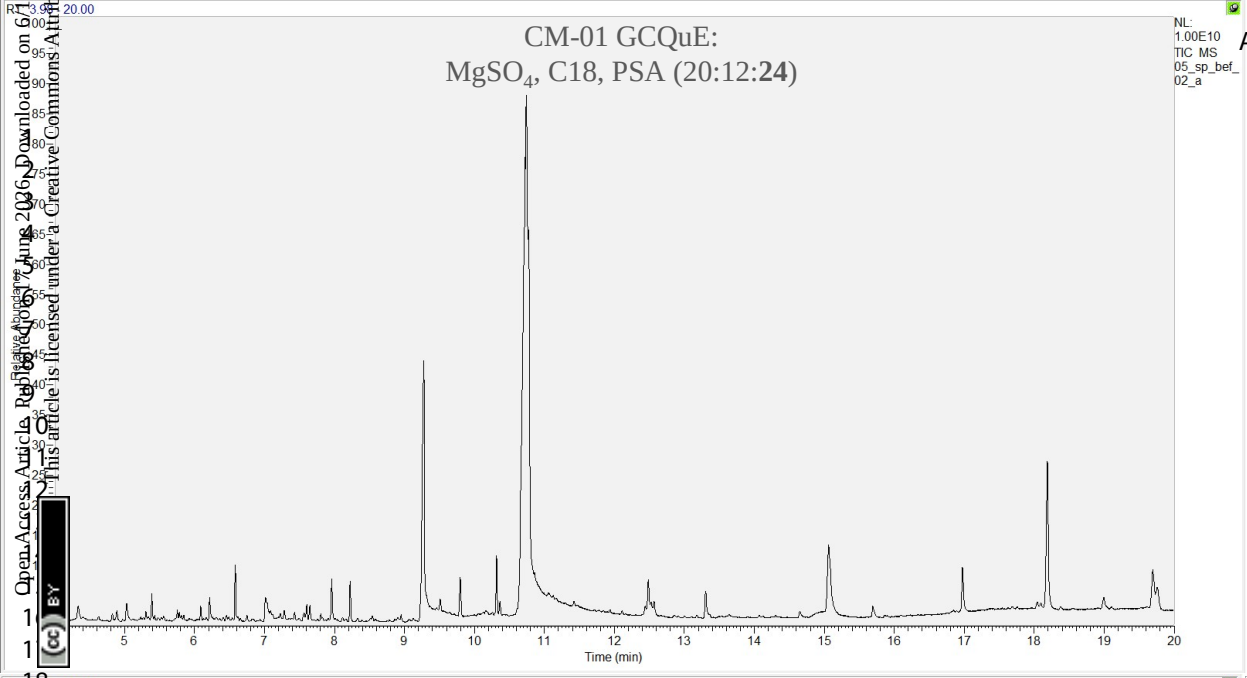
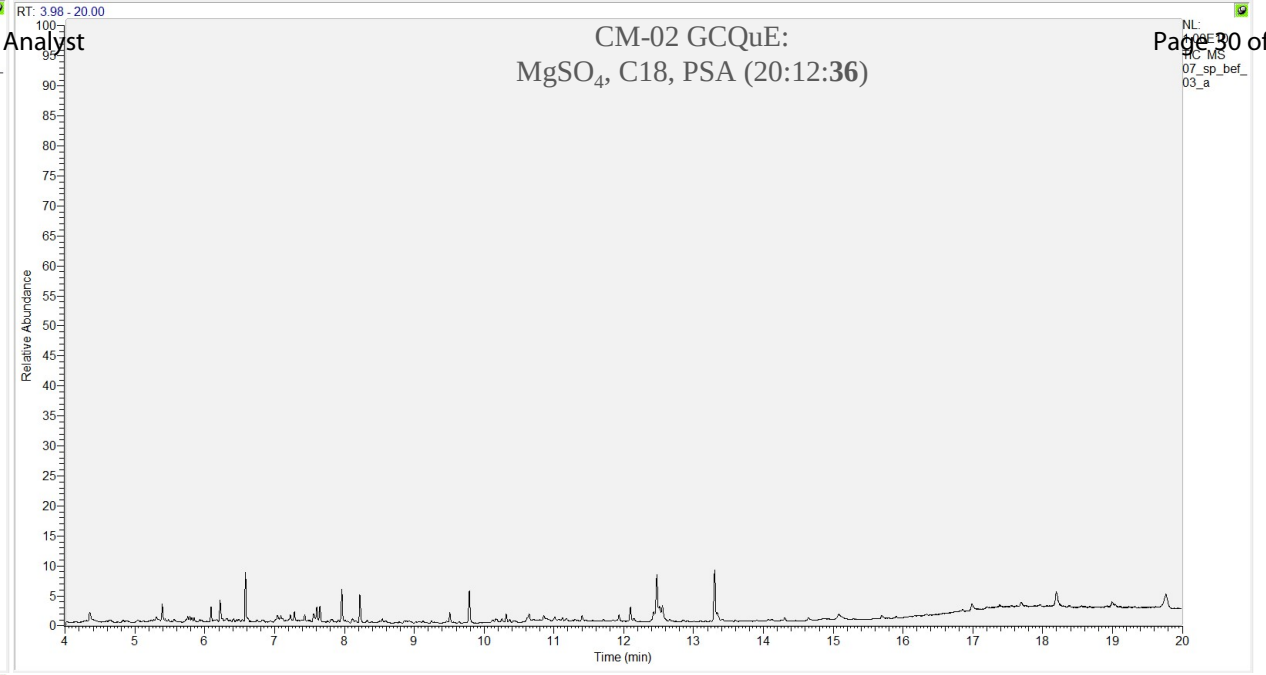
Table 2. Number of pesticides with clean-up recoveries >70%, <70%, and not detected (ND) for the selected matrices using the different μ-SPE cartridges.

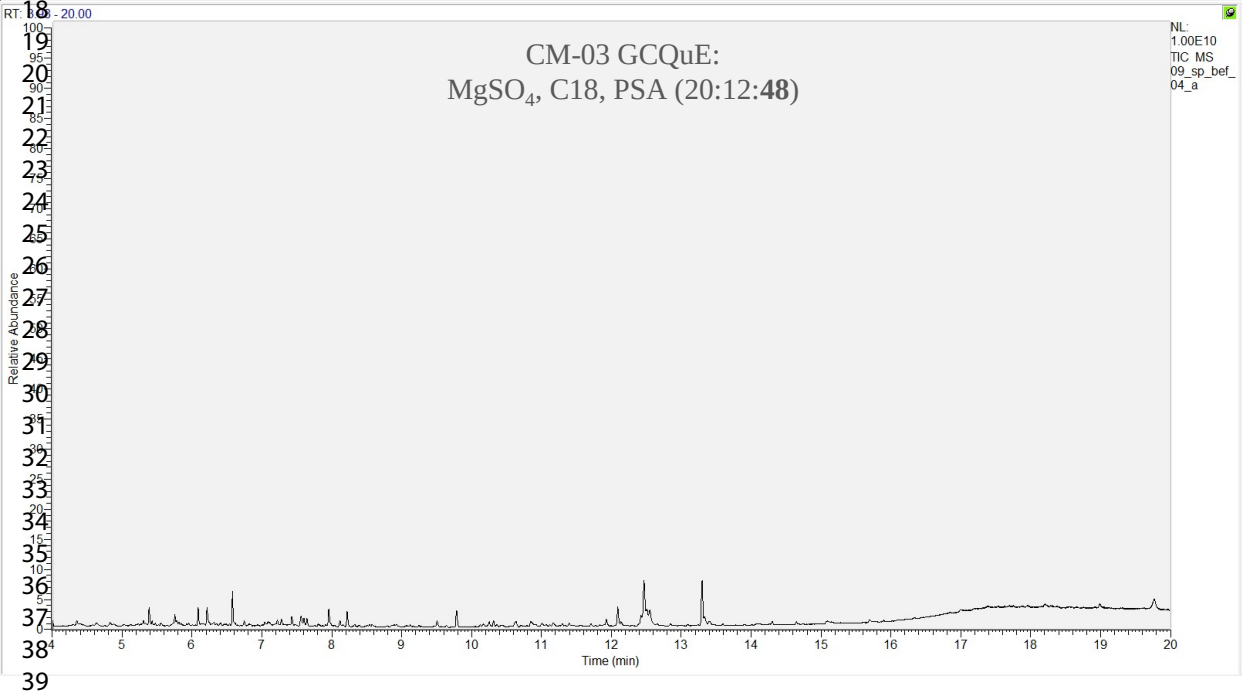
	Wheat			Linseed		
Cartridges Type	**Rec >70**	**Rec <70**	**ND**	**Rec >70**	**Rec <70**	**ND**
CM-01 GCQuE	62	0	0	59	2	1
CM-02 GCQuE	55	2	5	47	6	9
CM-03 GCQuE	53	3	6	51	0	11
GCQuE1-45	61	0	1	60	2	0

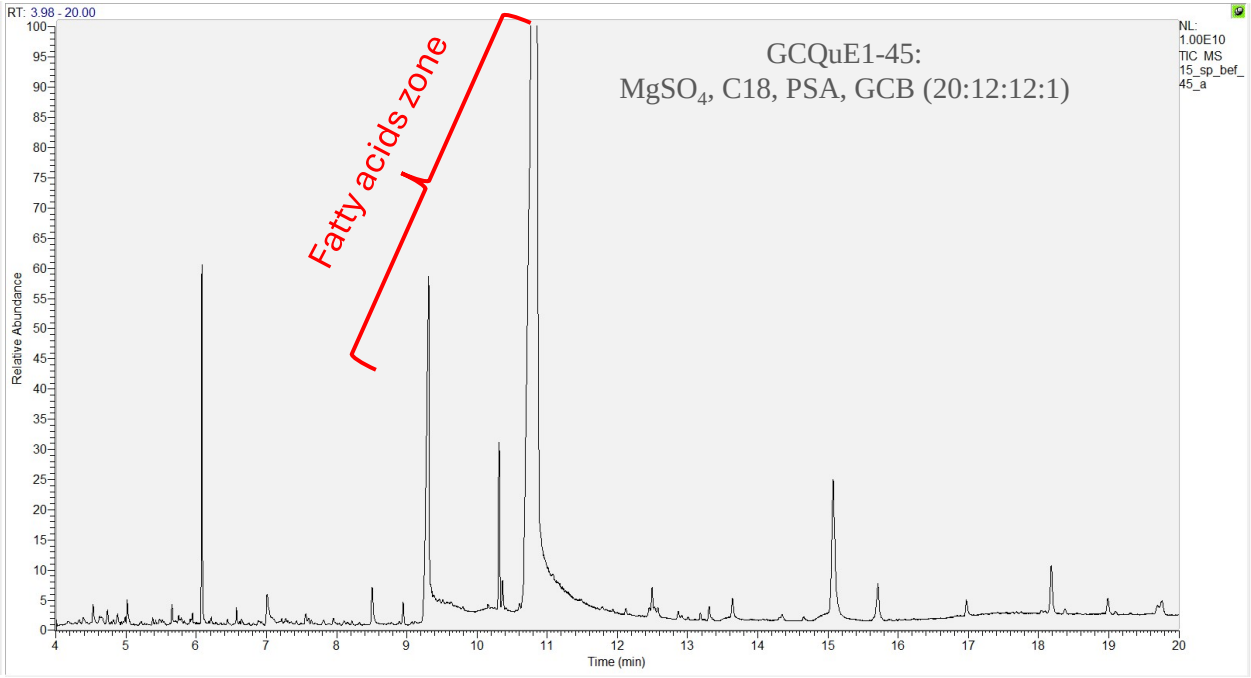


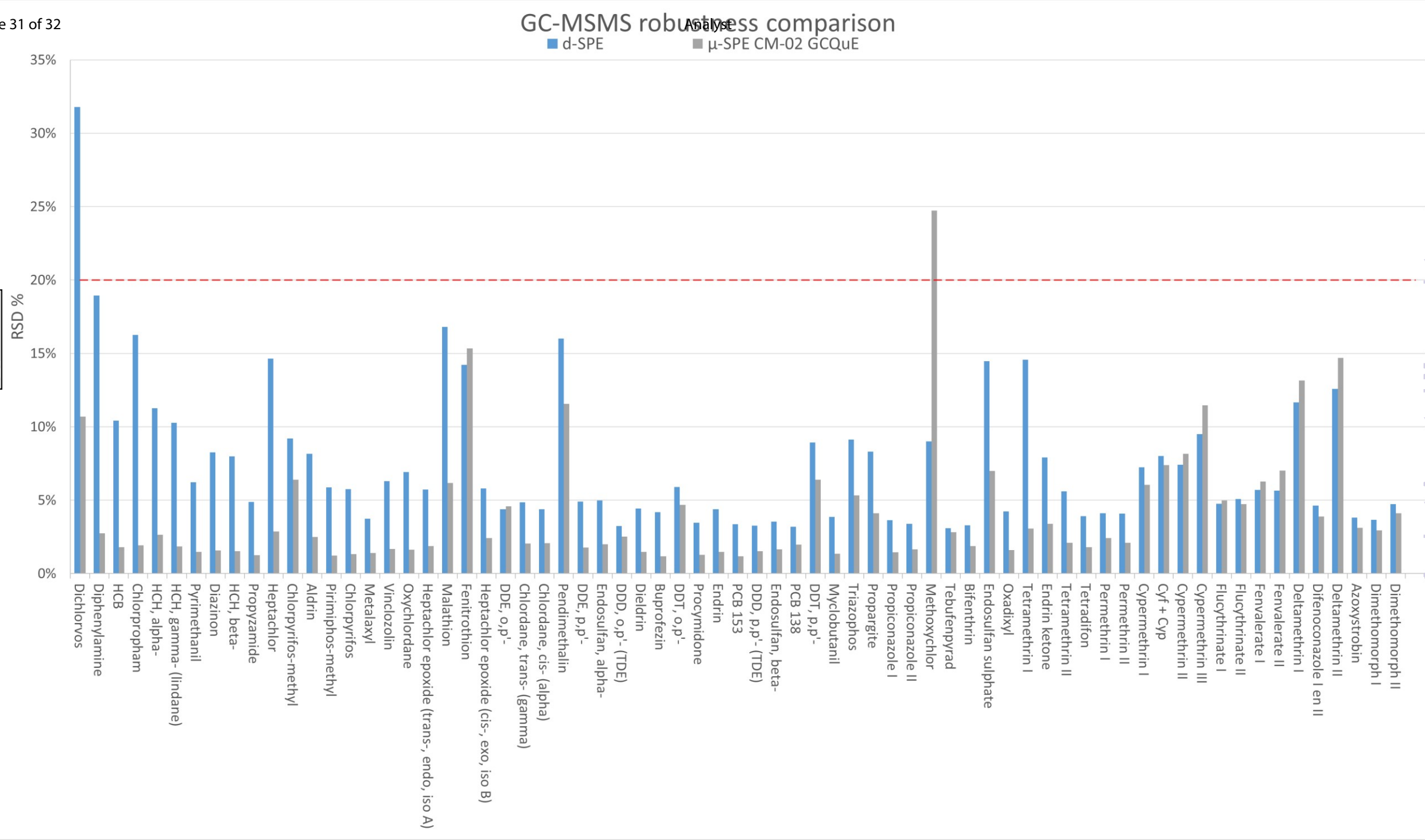












Data availability

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All data is reported in the article and provided in the supporting information files.