Testing the efficiency of extraction of incurred residues from soil with optimized multi-residue method

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Abstract

~~The efficient extraction of pesticide residues from various matrices is of primary importance for obtaining unbiased results.~~ The reproducibility of extraction of residues from spiked soil samples and from soils containing incurred residues was tested with ^{14}C-labeled test compounds of different physical-chemical properties. Nearly 100% of the compounds added to the sample before extraction could be recovered with an average reproducibility relative standard deviation (CV) of 5.4%. The additional steps of the determination process (cleanup, evaporation, etc.) contributed to the major part of the variability of the results (CV=10-20%). The incurred residues were most efficiently extracted with acetone for 30 min followed by the mixture of acetone/ethyl acetate 1:1 for additional 30 min. However, they could only be recovered at various extent (64-90% of total residues), underlying the importance of testing the efficiency of extraction. The residues were identified and quantified by gas chromatography applying thermionic detector. The performance parameters of the method complied with the international method validation guidelines, and they proved to be robust and suitable for determination of pesticide residues in soils of widely different physical-chemical properties.

Keywords: residue analysis, pesticide residues in soils, efficiency of extraction, incurred residues.

Introduction

There are several extraction methods for determining pesticide residues in soil. Traditionally the Soxhlet [1, 2] and the solid phase (SPE) extractions [3-6] are used as official methods in many countries. Their main drawbacks are requiring large volume of solvents, and the lengthy extraction time. Among the new techniques, the supercritical fluid extraction (SFE), [2,7, 8] pressurized solvent extraction (PLE), [9, 10] accelerated solvent extraction (ASE), [11, 12] microwave-assisted solvent extraction (MASE, MAE) [12-17] are the most widely used methods for extraction of pesticides from environmental samples. These methods produce high recovery of residues applying specific expensive instruments and large solvent volumes in some cases. Ultrasonic solvent extractions (USE) is one of the preferred techniques [12,18-22] as it can be performed with less solvents and shorter time. The solid phase micro extraction (SPME) [6, 17, 23] is mainly used for determining volatile compounds.

Wide range of solvents have been used depending on the purpose of the analysis. Acetone or acetone – water mixture is frequently used [18, 20, 23-25] in which the soil clods fall apart facilitating the complete partition of compounds between the soil and solvent phase. Disintegration of soil particles is further assisted by adding ammonium chloride (NH_4Cl), ammonium phosphate $(NH_4)_3PO_4$.[26-28] The mixtures of acetone with hexane, ethyl acetate (EtAc) or toluene improve the recovery of compounds of wide polarity range. [1, 9, 16] Previously dichloromethane was also used, [24, 25] but currently its use is restricted for protecting the environment. For its replacement EtAc and cyclohexane are applied. [12, 19, 20, 29] The presently applied methods are the variants of those used for residue analysis in food matrices, such as the QuEChERS method [12, 28, 30, 31] involving acetonitril for extraction. The

EN 12393-2:2014 Standard, [32] based on acetone or EtAc/cxyclohexane 1:1 solvent extraction, was also applied for soil matrices.[12]

The methods should be validated before use to provide evidence that they fit for the intended purposes. [33, 34] The generally acceptable main performance parameters are: specificity: signal resulted from untreated control sample is less than 30% of limit of quantification (LOQ) which is the lowest concentration that can be quantified reproducibly with known uncertainty; [35] sensitivity (LOD): typically 0.2 [36] - 0.3 LOQ set generally at 10 times the noise level; [37] matrix effect <±20% compared to response of pure standard solution; the mean recovery: within 70-120%. [34] The linearity and goodness of calibration should be tested with minimum 5-point calibration covering the analytical range. Its measure is the standard deviation of relative residuals [38] (S_{rr}) instead of the usually applied coefficient of regression (R^2).

$$S_{rr} = \sqrt{\frac{\sum(Y_{rel,i} - \bar{Y}_{rel})}{n-2}} \quad (1)$$

$$Y_{rel}\% = 100 \times \frac{Y_i - \hat{Y}}{\hat{Y}} \quad (2)$$

Where Y_i is the response and $\hat{Y}_i$ is read from calibration line for x_i calibration concentration, n is the number of ≥5 calibration points. Since the standard deviation of the residuals is usually proportional to the injected analyte, the standard deviation of the relative residuals reflects the average variability of the calibration points. Applying weighted linear regression S_{rr} should be ≤ 20%. [34]

The uncertainty of the measured residue values should be ≤ 20%. It is usually expressed as the relative standard deviation obtained from repeatability and within laboratory reproducibility determined from minimum 5 recovery studies.

Though the Codex Method Validation GLs [33] and the FAO/WHO Joint Meeting on Pesticide Residues (JMPR) [39] list the efficiency of extraction and homogeneity of analytical sample obtained from the laboratory sample as a basic performance characteristics to be tested, the published validation reports rarely include these important parameters. It should be emphasized that neither the analyses of proficiency tests and collaborative study samples nor recovery studies performed with spiked test portions removed from the analytical sample provide information on the homogeneity of analytical sample. The efficiency of extraction can only be determined from these studies, if the samples contain incurred residues.

The objectives of our study are to test the applicability of widely used solvents (acetone, ethyl acetate and hexane) that can be used with GC-NPD (nitrogen phosphor selective detector) and ECD if GC-MS/MS or LC-MS/MS systems are not available for determination of pesticide residues, optimize the extraction procedure and assess its efficiency for extraction of ^{14}C-labeled incurred residues from soil.

Materials and methods

Equipment

In addition to the usual laboratory glassware and devices, the following major equipment was used: Beckman 6000 TA liquid scintillation counter (LSC) with automatic quenching compensation; OX400 Biological Oxidizer; Stephan UM 5 Universal and Tecator 2096 laboratory homogenizers; Sigma 4K15 centrifuge; Mettler top load (0.01 g) and analytical (0.00001 g) balances; Edmund Bühler SM 25 and Certomat SII sieve shakers; TurboVap (Zymark) solvent evaporator; Varian 3800 gas chromatograph equipped with thermoionic

(TSD) detector and PTV injector (1079); CP-Sil-8CB Low Bleed MS column (25m × 0.32mm, df = 0.25µm); and 2.5m × 0.32mm methyl deactivated retention gap. Aglient GC with split/splitless injector and nitrogen, phosphor sensitive detector.

Materials

Ultima Gold^{TM} liquid scintilation coctail (Perkin Elmer) for LSC;

Calibration Standard for LSC**:** normal activity of ^{14}Carbon standard (code CRF 101, Amersham International plc, UK) is 5000 disintegrations per minute (dpm). ^{14}C radionuclide purity >99.9%. Half-life 5730 ± 40 years.

Absorption Solution for $^{14}CO_2$ for biological oxidizer**:** 10 mL of ethanolamine/methanol (12.5/87.5, v/v).

Filter paper to determine blank background activity of biological oxidizer.

Anhydrous calcium chloride (Merck reag. grade).

Analytical reference standards >98% purity (Dr. Ehrenstorfer GmbH): azinphos-ethyl (S-(3,4-dihydro-4-oxobenzo[d]-[1,2,3]-triazin-3-ylmethyl) O,O-diethylphosphorodithioate), chlorfenvinphos (2-chloro-1-(2,4-dichlorophenyl)ethenyl phosphate), chlorpyrifos (O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate), dimethenamid ((RS)-2-chloro-N-(2,4-dimethyl-3thienyl)-N-(2-methoxy-1-methylethyl)acetamide), oxyfluorfen (2-chloro-α,α,α-trifluoro-p-tolyl 3-ethoxy-4-nitrophenyl ether), pendimethalin (N-(1-ethylpropyl)-2,6-dinitro-3,4-xylidine), promertyn (N^2,N^4-diisopropyl-6-methythio-1,3,5-triazine-2,4-diamine), propazine (6-chloro- N^2,N^4-diisopropyl-1,3,5- triazine-2,4-diamine), terbuthylazine (N^2-tert-butyl-6-chloro-N^4-ethyl-1,3,5-triazine-2,4-diamine), terbutryn (N^2-tert-butyl-N^4-ethyl-6-methylthio-1,3,5-2,4-diamine).

^{14}C-labeled reference standards: triazol-ring- ^{14}C-atrazine (6-chloro-N^2-ethyl-N^4-isopropyl-1,3,5-triazine-2,4-diamine) (specific activity 1.6 Mbq/mg radioactive purity 96.5%, provided by Syngenta, 96,5%), (2,2-dimethyl, 3)-^{14}C-carbofuran (CA) (2,3-dihyro-2,2-dimethylbenzofuran-7-yl methylcarbamate), ethyl-1-^{14}C-chlorfenvinphos (CF), Ethyl-1- ^{14}C-chlorpyrifos (CP) and ethyl-1-^{14}C-p,p'-DDT (DT) (1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane) >95% radioactive purity provided by the International Atomic Energy Agency (IAEA).

The characteristic physical properties and chemical structural formula indicating the label position(s) are summarized in Table 1 and shown in Figure 1, respectively.

Soils used in the experiments

About 20 kg soil was collected from the top 15 cm layer at six sites having different physical characteristics (Table 2.) which could affect the recovery of the pesticide residues.

The samples were prepared following the ISO 11464:2006 Standard[40] and processed as described by Suszter et al. [41] The two terms are synonyms, but in pesticide residue analysis they indicate different operations.

Sample preparation: the procedure used, if required, to convert the laboratory sample into the analytical sample, by removal of parts (soil, stones, bones, etc.) not to be included in the analysis. [33]

Sample processing: the procedure(s) (e.g. cutting, grinding, mixing) used to make the analytical sample acceptably homogeneous with respect to the analyte distribution, prior to removal of the analytical portion. The processing element of preparation must be designed to avoid inducing changes in the concentration of the analyte. [33]

152

153 ***Methods***

154

155 *Determination of dry matter content of soil samples*

156

157 Clean porcelain dishes were pre-heated at 105 °C until constant weight (c [g] and stored over
158 activated anhydrous $CaCl_2$ in desiccator until use. Ten g of processed and homogenized
159 sample were weighted to the porcelain dishes (a [g]) and heated at 105 °C until constant
160 weight, cooled to room temperature in desiccator and weighted again (b [g]. The dry matter
161 content (dm [%]) of the soils was calculates as:

162 $$dm = 100 - 100 \times \frac{a-b}{a-c} \quad (3)$$

163 All residue values were expressed on dry matter basis in this study.

164

165 *Determination of ^{14}C activity of samples.*

166

167 Before the series of radioactivity measurements were started, the efficiency of the biological
168 oxidizer, used for determining the ^{14}C activity in soil samples, was tested. Ten mL of
169 absorption solution and 5 mL of scintillation cocktail were pipetted into a scintillation vial to
170 absorb the evolved $^{14}CO_2$. First the background activity was measured by placing about 500
171 mg filter paper into the combustion boot followed by the measurement of the activity of a
172 complete strip of ^{14}C standard paper for calibration. The absorbed ^{14}C activity was measured.
173 The efficiency of the oxidizer was calculated as:

174

175 $$Efficiency = \frac{dpm\ standard\ after\ combustion - dpm\ blank\ after\ combustion}{dpm\ standard} \quad (4)$$

A recovery of 98% or greater indicated that the oxidizer worked efficiently.

The ^{14}C activities of the reference standard, the background activity of soil, as well as the treated soils (500 mg) were determined following the same procedure.

For the determination of the ^{14}C activity of the extracts, 12 mL scintillation cocktail and 5 mL extract were transferred into 20 mL LSC vial. The vial was tightly closed, shaken and placed in the Beckman LSC counter.

Each sample was measured three times for 5 minutes, after running the self-calibration program, and their average activity was used for further calculations. The average relative standard deviation of the replicate LSC measurements of 29 test portions was 0.0073.

Determination of the reproducibility of extraction of spiked samples

Six soils of different physical-chemical properties (U129, V01, V02, W33, X65, and Y97) shown in Table 3 were used for studying the reproducibility of extraction.

For the treatment of soil test portions, an acetone stock solution containing ^{14}C-labeled atrazine and cold atrazine analytical standard at 0.05 mg/mL concentration with 50,000 Bq/mL (3,000,000 dpm/mL) target specific activity was prepared.

Test portions of 20 g of processed soil samples were weighed into Petri dish. One thousand µL of 0.001mg/mL atrazine standard solution, prepared from the stock solution, was spread over the soil surface with Hamilton syringe (spike level 0.05 mg/kg). The spiked sample was kept in fume hood for 30 min to evaporate the acetone, then transferred into a 250 mL centrifuge tube and 2.8 mL of 0.2 mol NH_4Cl solution and 40 mL acetone were added. The tightly closed tube was shaken for 30 min at 200-250 rpm with Certomat SII shaker. The

200 shaking frequency was selected to keep the whole amount of soil continuously moving. The
201 soil and the extract was separated with centrifuging (Sigma 4K15) at 3000 rpm.
202
203 Based on the accurate weights of soil and spiking solutions and the latter one's measured
204 activities, the expected activities (A_{spike}) were calculated. The recovery of the residue (Q) was
205 calculated from the average of the three replicate ^{14}C activity measurements of the extract
206 (A_E) taking into account the background activity of the soil sample (A_0).

207 $$Q = \frac{A_E - A_0}{A_{spike}} \quad (5)$$

208 Test portions of each soil sample were spiked and extracted by different analysts 4 or 5 times.
209 The standard deviation of the recovery (S_Q) values, obtained from the repeated tests, was
210 calculated and divided by the average of recoveries to obtain the reproducibility relative
211 standard deviation ($CV_{QR}=S_Q/\bar{Q}$).
212 The combined uncertainty of extraction based on all results (n) of testing the five different soil
213 samples was calculated from the pooled variances (S_Q^2) and the grand average of recoveries
214 ($\bar{\bar{Q}}$):

215 $$CV_e = \frac{\sqrt{\frac{1}{n}\sum_{i=1}^{n} S_Q^2}}{\bar{\bar{Q}}} \quad (6)$$

216
217
218 *Determination of the efficiency of extraction*
219
220 The efficiency of extraction was tested with four ^{14}C-labeled pesticide (carbofuran,
221 chlorfenvinphos, chlorpyriphos and p-p-DDT) and 3 different soils (V01, V02 and X65). The
222 test compounds were prepared separately in acetone containing the ^{14}C-labelled (target
223 specific activity 400 Bq/mL (24000 dpm/mL) and unlabeled standards in 0.3 µg/mL

concentration. The exact activities of the standard solutions were determined with LSC in three replicates.

Twenty grams of processed soils were weighed in 250 mL round bottom flask and treated with 30 mL acetone containing the standard solution at 0.05 mg/kg dry soil equivalent. Each standard solution was applied to different portions of soil. The flask was fixed on rotary evaporator and rotated for 15 minutes at ambient temperature to thoroughly mix the soil and the solvent, then the solvent was evaporated under gentle vacuum immersing the flask into water bath kept at 35ºC. The dry, free flowing soil powder was transferred to 100 mL centrifuge tube with screw cap. Distilled water was added until water holding capacity of the soil and the container was stored in the greenhouse of the IAEA at about 25ºC for 6 months. The evaporated water was replaced regularly. Twelve replicates were prepared from each of the soil-pesticide combinations. Untreated soils were processed similarly to fortified ones and they were used to determine the background activity. They also served as blank sample for validation of the optimized method.

The exact initial ^{14}C activities of the fortified soils containing the incurred residues and the blank soils were determined just before their extraction, as described above.

After 6 months of storage, the soil samples were extracted with either of hexane:acetone (1:1 v/v), acetone and ethyl acetate (EtAc). The extracting solvents were selected from those which have been most frequently used for determination pesticide residues in soil and plant materials. Dichloromethane was not considered in view of protection of the environment. The three solvents have high and medium polarity and non-polar character. They were primarily suitable for extraction of pesticides of similar polarity.

The extraction procedure, performed in 3 replicates, consisted of 4 steps:

1. Before extraction, 2.8 mL 0.2 mol NH_4Cl was thoroughly mixed with the 20 g soil sample and let to stand for 15 minutes, then 40 mL of one of the extraction solvents was added, the container was tightly closed and agitated with horizontal shaker at 200-250 rpm for 30 min.
2. The tube was centrifuged at 3000 rpm, 1-1 mL of clean extract were withdrawn and mixed with 12-12 mL scintillation cocktail in LSC vials. The radioactivity was determined for 3×5 minutes with Backman LSC counter.
3. The tube was agitated again with horizontal shaker, for another 30 mins (total extraction time 1 hr)
4. Step 2 was repeated and the extraction was continued for another hour (total extraction time 2 hours).

The radioactivity of the extract was measured after 30, 60 and 120 minutes.

Based on the results of the first series of tests, an additional extraction procedure was tested: the 20 g soil was first extracted with 20 mL acetone, then 20 mL ethyl-acetate was added and the agitation of the soil was continued for 30 mins (total extraction time 1 hour). The use of combination of solvents was necessary, because acetone completely disintegrated the soil particles which increased the efficiency of extraction and the ethyl acetate extracted non-polar residues as well.

The extracting solvent was decanted after the end of the extraction. The soil was rinsed with 20 mL extracting solvent, centrifuged, the supernant solvent was decanted and the soil was kept under fume hood until constant weight was reached. The radioactivity of the extracted soil was determined from 500 mg portions.

All measured residue concentrations were expressed on dry soil basis.

Description of optimized analytical procedure

Azinphos-ethyl, dimethenamid, chlorfenvinphos, chlorpyrifos, oxyfluorfen, pendimethalin, promertyn, propazine, terbuthylazine and terbutryn were selected as test compounds representing wide range of water solubility, volatility and octanol – water partition coefficient (supplementary information Table S1) like those of ^{14}C-labeled test compounds used for studying the efficiency of extraction (Table 1).

The untreated soils were spiked with the mixtures of standard solutions at concentration levels equivalent to LOQ, 20LOQ and 100LOQ. Three different types of soil samples (X65, V01 and V02) were processed with adding sufficient water as described by Suszter et al. [41] Twenty grams of processed soil was weighed into centrifuge tube, 2.8 mL 0.2 mol NH_4Cl was added and mixed with the soil with a glass road. Twenty mL acetone containing 120 ng chlorpyrifos/mL internal standard (ISTD) was added, the tube was tightly closed and agitated on a horizontal shaker at 200 rpm for 30 minutes. Twenty mL ethyl acetate was added to the extract and the shaking was continued for 30 minutes. The soil was let to settle and the tube was centrifuged at 3000 rpm. Ten mL extract (equivalent to 5 g soil) was pipetted into 20 mL test tube, it was dried by shaking with 6 ± 0.1 g anhydrous sodium sulfate for 20 seconds, and transferred into a 20-mL calibrated glass test tube through filter paper inserted in a glass funnel. The centrifuge tube and the filter funnel was rinsed with 3×2 mL EtAc. The solvent was evaporated with nitrogen to about 0.5 mL with TurboVap®VL evaporator at maximum 30 ^{o}C and 1 psi pressure. The final volume was adjusted exactly to 2 mL. No further cleanup was employed.

The qualitative and quantitative determination of the residues was carried out with Varian 3800 GC equipped with nitrogen and phosphorus selective thermionic detector (TSD) and

PTV injector. Aglient 7890A GC with NPD was used for confirmation of the identity of the analytes. The chromatographic conditions are summarized in supplementary information (Table S2). The condition of the chromatographic system (resolution, phosphorus-carbon and nitrogen-carbon selectivity, peak asymmetry, stability of retention times) was checked by injecting the system suitability test mixture [38] at the beginning and at the end of each batch of chromatographic analyses of sample extracts. An example chromatogram of the SST mixture is shown in Figure 2. If the system suitability test indicated malfunction the appropriate maintenance actions were taken.

The matrix effect was compensated by preparing the calibration standard solutions from the blank soil extracts. The concentration of the test compounds in the calibrating standard solutions ranged from 0.5 LOQ to 150 LOQ. The weighted linear regression lines, based on 5 concentration points, and their confidence intervals, as well as the S_{rr} values were calculated with a self-made Excel template. Examples for typical calibration charts are given in Figure 3. The LOD, LOQ, RT and RRT are summarized in Table 3.

The specificity of the detection was checked with injecting the standard mixture, extracts of the soil and reagent blanks. The specificity was acceptable if no interfering peak was larger than 0.3LOQ. Examples for the three chromatographic runs are shown in Figures 4 and 5.

The compounds were identified based on their retention times relative to chlorpyrifos ISTD (RRT) (Table 3). The ratio of peak areas of analytes and ISTD were evaluated with Star 6.2 software for the quantitative determination.

Results and discussion

Reproducibility of extraction

It was tested on five different soils with ^{14}C-atrazine at spike level of 0.05 mg/kg. Four or five test portions from each soil was spiked and extracted with acetone on different days by 3 analysts. The radioactivity of each extract was measured 3 times for 5 min with Beckman LSC. The recovery of ^{14}C-atrazine was calculated with equation 5 from the average recovered activity and the activity of the spiking solution. The potential outliers were tested with Grubb's and the homogeneity of variances with Cochran tests. [42] The average recoveries obtained from 28 independent recovery tests performed with the 6 different soils were compared with analysis of variances (ANOVA) [42], however none of the neighboring ones, in the rank ordered values, differed more than the least significance difference (0.249) indicating that there was no difference in the recoveries from different soils. Consequently, the grand average of recoveries could be calculated from all recovery data. The calculation of the reproducibility of extraction (CV_{eQ}) is shown with an example in Table 4. The reproducibility of extraction of 5 types of soil samples was calculated by pooling the CV_e values obtained with different soils. The results, indicating complete recovery (100.9%) of all tests with a pooled CV_{extr} (0.0054), are summarized in Table 5.

Efficiency of extraction of incurred residues

The ^{14}C-activites derived from incurred residues of carbofuran, chlorfenvinphos, chlorpyriphos and p-p-DDT were determined in three different soils (V01, V02 and X65) after 6-month storage at about 25 ^{o}C.

In some cases, the recovered ^{14}C activity has remained practically the same after 30 minutes. However, in other cases the recovery improved (DDT–EtAc, chlorpyrifos–acetone, chlorfenvinphos–EtAc) significantly if the shaking was continued for 60 minutes, and remained practically constant afterwards. Acetone completely disintegrated the soil particles, while in case of hexane and EtAc some clods were formed or remained in the extracted soil. During the extraction with acetone-hexane mixture two phases were formed. The upper hexane-acetone phase contained mainly the non-polar compounds, while the polar compounds partitioned into the lower (acetone−water) phase, which is not desirable for quantitative determination of residues.

Based on the experience gained with various solvents and extraction time, we concluded that starting the extraction with acetone for 30 mins, adding EtAc and continuing the shaking for another 30 minutes would give the highest recoveries for pesticide residues having wide range of polarity. The optimal proportion of soil extracting solvent was not tested, but taken from many publications applying the soil/solvent ratio of 1:2. Taking into account the vast experience with the application of QuEChERS method [43] acetonitrile would be a generally applicable solvent for extracting residues from soil [28, 30, 31] if MS detection would be used, however acetonitrile cannot be directly used with N-P selective thermoionic and electron capture detectors, therefore its applicability was not tested.

The remaining activities in the extracted soil was measured after the combined acetone−EtAc extraction procedure. The results, summarized in Table 6, show that the proportion of ^{14}C activity in the soil varied in different pesticide−soil combinations.

As the adsorption of pesticide residues to soil particles and their partition between soil−extracting solvent depend on the combination of several physical-chemical properties of soil, the number of tests and combinations did not allow detailed analyses of their

relationship. Nevertheless, our experiments clearly indicate the importance of testing the efficiency of extraction with incurred residues as part of the validation or extension of the scope of a method. The most convenient way of testing the efficiency of extraction is to use ^{14}C-labeled test compounds, but they are not readily applicable in routine pesticide residue laboratory. Therefore, the Codex GLs on method validation [33] and the FAO JMPR Manual [39] provide some generally applicable alternative procedures.

It is emphasized that the measured activities include the parent compound and its metabolites which contain ^{14}C. The proportion of parent compound and metabolites depends on several factors such as the time between pesticide application and sampling, microbiological activity, pH and physical properties of soil, therefore the concentration of the parent compound would be lower than that indicated by ^{14}C measurement.

Applicability of optimized procedure for analysis of pesticide residues in soil

The test mixture of 10 pesticide active substances having wide range of physical-chemical properties (Table S1) were used to spike 3 different types of soils at 3 concentration levels of 100-fold difference. The linearity of the response of components of the standard mixture was established in the range of 0.5 LOQ and 150LOQ. The goodness of calibration, was characterized by the coefficient of regression (R^2) and the standard deviation of relative residuals (S_{rr}). Both parameters were well within the acceptable range specified by the European Union Quality control guidance document. [34]

The reproducibility of determination of residues from spiked samples was tested with 5 replicates in each soil and spike level. The results revealed that there was no difference among the reproducibility of analyses depending on the type of soil, which is in line with the findings of reproducibility of extraction. The average recoveries ($\bar{Q}_{L1}$) and reproducibility relative

standard deviations (CV_Q), summarized in Table 7, are within the acceptable limits of the corresponding quality control guidelines.

CONCLUSIONS

Use of ^{14}C-labeled compounds enabled quantifying the analytes present in the LSC cocktail with an average 0.0073 relative uncertainty. Our results proved that the residues can be extracted from spiked soil samples with an average CV_e of 0.54%. The major part of the variability of results of residue analysis derived from the further steps (evaporation, cleanup and instrumental analyses), which may require special attention if the combined relative reproducibility uncertainty of the results is getting close to the upper acceptable limit of 25%. [34] The efficiency of extraction depends on several factors and up to about 35% of total residue might remain unextracted which can lead to biased results. The recovery tests performed with spike samples do not reveal the required information. Therefore, the efficiency of extraction should be tested with incurred residues in every case when a new extraction procedure is validated or an established method is extended to a new matrix. For this purpose, alternative methods [39] are available if the application of ^{14}C-labelled compounds is not feasible.

In the lack of GS-MS/MS, LC-MS/MS instruments, the GC with specific detectors and appropriate cleanup procedures can be reliably used for determination of pesticide residues especially in samples of know pesticide treatment history, or in selective field surveys targeted for specific pesticide residues.

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REFERENCES

[1] EPA Method No.1699. Pesticides in water, soil, sediment, biosolids and tissue by *HRGC/HRMS*. 2007.

[2] Schneider, R. Evaluation of an extraction method for triazine herbicides from soils for screening purposes. Agribiol. Res. **1995**, *48*, (3-4), 193-206.

[3] Picó, Y.; Fernández, M.; Ruiz, M.; Font, G. Current trends in solid-phase-based extraction techniques for the determination of pesticides in food and environment. J. Biochem. Biophys. Methods, **2007**, *70*, 117-131.

[4] Lourencetti, C.; De Marchi, M.; Ribeiro, M. Determination of sugar cane herbicides in soil and soil treated with sugar cane vinasse by solid-phase extraction and HPLC-UV. Talanta, **2008**, *77*(2), 701-709.

[5] Dabrowska, H.; Dabrowski, L.; Biziuk, M.; Gaca, J.; Namies'nik, J. Solid-phase extraction clean-up of soil and sediment extracts for the determination of various types of pollutants in a single run. J. Chomatogr. A, **2003**, *1003*(1-2), 29-42.

[6] Lambropoulou, D.; Konstantinou, I.; Albanis, T. Factors affecting multiresidue determination of priority herbicides when using solid-phase microextraction. J. AOAC Int. **2002**, *85*(2), 486-493.

[7] Gonc Alves, C.; Carvalho, J.; Azenha, M.; Alpendurada, M. Optimization of supercritical fluid extraction of pesticide residuesin soil by means of central composite design and

analysis by gaschromatography–tandem mass spectrometry. J. Chromatogr. A, **2006**, *110*. 6-14.

[8] Kreuzig, R.; Koinecke, A.; Bahadir, M. Use of supercritical fluid extraction in the analysis of pesticides in soil. J. Biochem. Biophys. Methods, **2000**, *43*, 403-409.

[9] Concha-Grana, E.; Turnes-Carou, M.I.; Muniategui-Lorenzo, S.; López-Mahía, P.; Fernández-Fernández, E.; Prada-Rodríguez, D. Development of pressurized liquid extraction and cleanup procedures for determination of organochlorine residues in soils. J. Chromatogr. A, **2004**, *1047*, 147-155.

[10] Hussen, A.; Westbom, R.; Megersa, N.; Mathiasson, L.; Björklund, E. Selective pressurized liquid extraction for multi-residue analysis of organochlorine pesticides in soil. J. Chromatogr. A, **2007**, *1152*. 247-253.

[11] Tao, S.; Guo, L.; Wang, X.; Liu, W. Use of sequential ASE extraction to evaluate the bioavailability of DDT and its metabolites to wheat roots in soils with various organic carbon contents. The Science of the Total Environment, **2004**, *320*. 1-9.

[12] Lesueur, C.; Gartner, M.; Mentler, A.; Fuerhacker, M. Comparison of four extraction methods for the analysis of 24 pesticides in soil samples with gas chromatography–mass spectrometry and liquid chromatography–ion trap–mass spectrometry. Talanta, **2008**, *75*, 284-293.

[13] Sun, L.; Lee, H. Optimization of microwave-assisted extraction and supercritical fluid extraction of carbamate pesticides in soil by experimental design methodology. J. Chromatogr. A., **2003**, *1014*, 165-177.

[14] Molins, C.; Hogendoorn, E.A.; Dijkman, E.; Heusinkveld, H.A.G.; Baumann, R.A. Determination of linuron and related compounds in soil by microwave-assisted solvent extraction and reversed-phase liquid chromatography with UV detection. J. Chromatogr. A, **2000**, *869*, 487-496.

[15] Kovačić, N.; Prosen, H.; Zupančič-Kralj, L. Determination of triazines and atrazine metabolites in soil by microwave-assisted solvent extraction and high pressure liquid chromatography with photo-diode-array detection. Acta Chim. Slov. **2004**, *51*, 395-407.

[16] Hogendoorn, E.; Huls, R.; Dijkman, E.; Hoogerbrugge, R. Microwave assisted solvent extraction and coupled-column reversed-phase liquid chromatography with UV detection Use of an analytical restricted-access-medium column for the efficient multi-residue analysis of acidic pesticides in soils. J. Chromatpgr. A, **2001**, *938*, 23-33.

[17] Shen, G.; Lee, H. Determination of triazines in soil by microwave-assisted extractionfollowed by solid-phase microextraction and gas chromatography–mass spectrometry. J.Chromatogr. A, **2003**, *985*, 167-174.

[18] Babic', S.; Petrovic', M.; Kaštelan-Macan, M. Ultrasonic solvent extraction of pesticides form soil. J. Chromatogr. A, **1998**, *823*, 3-9.

[19] Gonc Alves, C.; Alpendurada, M. Assessment of pesticide contamination in soil samples from an intensive horticulture area, using ultrasonic extraction and gas chromatography–mass spectrometry. Talanta, **2005**, *65*, 1179-1189.

[20] Tor, A.; Aydin, M.; Özcan, S. Ultrasonic solvent extraction of organochlorine pesticides from soil. Analytica Chimica Acta, **2006**, *559*, 173-180.

[21] Lambropoulou, D.; Albanis, T. Determination of the fungicides vinclozolin and dicloran in soils using ultrasonic extraction coupled with solid-phase microextraction. Analytica Chimica Acta, **2004**, *514*. 125-130.

[22] Sánchez-Brunete, C.; Rodriguez, A.; Tadeo, J. Multiresidue analysis of carbamate pesticides in soil by sonication assisted extraction in small columns and liquid chromatography. J. Chromatogr. A, **2003**, *1007*, 85-91.

[23] Redondo, M.; Ruiz, M.; Boluda, R.; Font, G. Determination of pesticides in soil samples by soild phase extraction disk. Chromatographia, **1993**, *36*, 187-189.

[24] Ambrus, A.; Lantos, J.; Visi, E.; Csatlós, I.; Sárvári, L. General Method for the Determination of Pesticide Residues in Samples of Plant Origin, Soil and Water, I Extraction and cleanup. J. Assoc. Off. Anal. Chem. **1981**, *64*, 733-742.

[25] Park, J-H.; Mamun, I.R.; Choi, J-C.; El-Aty, A.M.A.; Assayed, M.E. Development of a multiresidue method for the determination of multiclass pesticides in soil using GC. Biomed. Chromatogr. **2010**, *24*. 893-901.

[26] Cayley, G.; Lord, A. K. The extraction and assay of thiabendazole in strong adsorbing soil. Pestic. Sci., **1980,** *11*, 9-14.

[27] Kihampa, C.; Ram Mato, R.; Mohamed, H. Residues of Organochlorinated Pesticides in Soil from Tomato Fields, Ngarenanyuki,Tanzania. J. Appl. Sci. Environ. Manage. **2010**, *14*(3), 37-40.

[28] Kadenczki, L.; Keresztény, N.; Szemánné Dobrik, H. Növényvédőszer-maradék analitikai multi-residue módszer kidolgozása talajmintákra a QUECHERS módszer átalakításával, GC/MS, LC/MS, GC/ECD és GC/FPD detektálással. I. rész: Foszforsavészterek. Műszaki Földtudományi Közlemények, **2012**, *83*(1), 101-110.

[29] Sánchez-Brunete, C.; Albero, B.; Tadeo, J. Multiresidue determination of pesticides in soil by gas chromatography-mass spectrometry detection. J. Agric. Food Chem., **2004**, *52*, 1445-1451.

[30] Yang, X.; Ying, G.-G.; Kookana, R. Rapid multiresidue determination for currently used pesticides in agricultural drainage waters and soils using gas chromatography–mass spectrometry. J. Environ. Sci. and Health, Part B., **2010**, *45*, 152-161.

[31] Fernandez, V.; Dominiques, V.; Mateus, N.; Delerue-Matos, C. Multiresidue pesticides analysis in soils using modified QuEChERS with disposablepipette extraction and dispersive solid-phase extraction. J. Sep. Sci., **2013**, *36*, 376-392.

[32] EN 12393-1:2004. Foods of plant origin. Multiresidue methods for the determination of pesticide residues by GC or LC-MS/MS. Part 2: Methods for extraction and cleanup, **2004**.

[33] Codex Alimentarius Commission. Guidelines on Good Laboratory Practice in Pesticide Residue Analysis, CAC/GL 40-1993 amended in **2000** https://www.google.hu/webhp?sourceid=chrome-instant&ion=1&espv=2&ie=UTF-8#q=GUIDELINES+ON+GOOD+LABORATORY+PRACTICE+IN+PESTICIDE+RESIDUE+ANALYSIS+CAC%2FGL+40-1993 (accessed Dec 2016).

[34] European Commission Directorate-General for Health and Food Safety (SANTE). Guidance document on analytical quality control and method validation procedures for pesticides residues analysis in food and feed. SANTE/11945/2015, **2015**, 19-20. http://ec.europa.eu/food/plant/docs/plant_pesticides_mrl_guidelines_wrkdoc_11945_en.pdf (accessed Dec 2016).

[35] Ellison SLR. A.; Williams A. Eds. EURACHEM/CITAC Guide: Quantifying Uncertainty in Analytical Measurement 3rd edition, ISBN 978-0-948926-30-3. 2012. https://www.eurachem.org/index.php/publications/guides/quam (accessed Dec 2016).

[36] Thompson, M.; Ellison, S.L.R.; Wood, R. Harmonized guidelines for single-laboratory validation of methods of analysis (IUPAC Technical Report), **2002**, *74*(5), 835-855.

[37] Codex Alimentarius Commission. Proposed draft guidelines on performance criteria for methods of analysis for the determination of pesticides residues, Appendix XI in Report of the 48th Session of the Codex Committee on Pesticide residues: Joint FAO/WHO Food Standards Programme, **2016**. http://www.fao.org/fao-who-codexalimentarius/sh-proxy/en/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcod

ex%252FMeetings%252FCX-718-48%252FReport%252FREP16_PRe.pdf (accessed Jan. 2017).

[38] Soboleva, E.; Ambrus A. Application of a system suitability test for quality assurance and performance optimisation of a gas chromatographic system for pesticide residue analysis J. Chromatography A, **2004,** *1027*, 55-65.

[39] Ambrus, Á. (ed.). FAO manual on the submission and evaluation of pesticide residues data for the estimation of maximum residue levels in food and feed. 3rd ed. FAO Plant Production and Protection Paper, **2016,** *225*, 32-33. http://www.fao.org/agriculture/crops/core-themes/theme/pests/jmpr/jmpr-docs/en/ (accessed Dec. 2016).

[40] ISO 11464:2006 Soil quality – Pretreatment of samples for physico-chemical analysis, Reviewed and confirmed in **2016**. http://www.iso.org/iso/catalogue_detail.htm?csnumber=37718 (accessed Dec 2016).

[41] Suszter, G.; Ambrus, A.; Schweikert-Turcu, M.; Klaus P.M., Estimation of efficiency of processing soil samples, J. Environ. Sci. and Health Part B, **2006**, *41*, 1-22.

[42] Miller J.N.; Miller J.C. *Statistics and Chemometrics for Analytical Chemistry* 6th ed. Pearson Education Ltd. Harlow, UK. 2010, 49-59.

[43] Anastassiades, M.; Tasdelen, B.; Scherbaum, E.; Stajnbaher, D. Recent developments in QuEChERS methodology for pesticide multiresidue analysis. In: *Pesticide chemistry: Crop protection, public health, environmental safety*. Ohkawa, H.; Miyagawa, H; Lee, P.W. Eds).; Wiley-VCH, Weinheim, 2007, Chapter XX.

FIGURE CAPTIONS

Figure 1. Structural formula indicating labeled positions of test compounds

Figure 2. Typical chromatogram of the system suitability test mixture containing EPTC, propoxur, tributyl-phosphate, dimethoate, pirimicarb, chlorpyrifos-methyl, parathion-methyl, carbaryl, chlorpyrifos, chinalphos, methidathion and phosalone.

Figure 3. Calibration charts of terbutryn on different days. The blue and red lines indicate the confidence and tolerance limits around the weighted regression line. Note the difference in R^2 and S_{rr}.

Figure 4. Example for specificity of detection of test compounds in extracts of Y97 soil (red colour), spiked at 20 LOQ level (Blue colour), and reagent blank green colour)

Figure 5. Example for specificity of detection of test compounds in extracts of X65 soil spiked at 20 LOQ level (red colour), bank extract (green colour), blank soil extract blue colour

TABLE CAPTIONS

Table 1. Physical properties of labeled compounds

Table 2. Summary of soil parameters

Table 3. Performance characteristics of GC determination of test compounds

Table 4. Example for the calculation of reproducibility of extraction

Table 5. Reproducibility of extraction of soil samples

Table 6. Efficiency of extraction of ^{14}C-labeled test compounds from soil

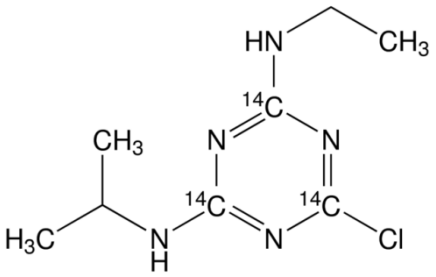
Table 7. Reproducibility of

SUPPLEMENTARY MATERIALS

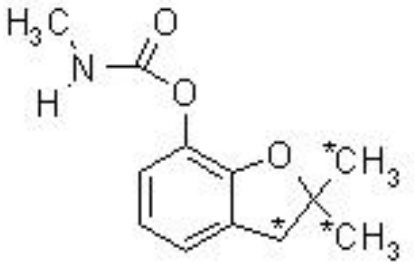
Table S1. Test compounds used for method validation

Table S2. Operation conditions of gas chromatographs

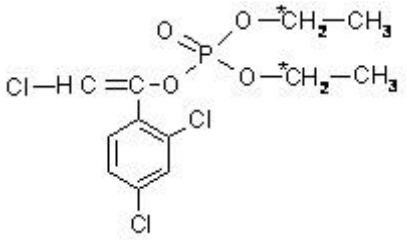
628 FIGURES



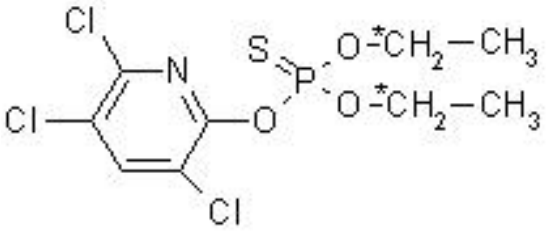
Atrazine [trazol ring-^{14}C]



Carbofuran [(2,2-dimethyl ,3)-^{14}C]



Chlorfenvinphos [ethyl-1-^{14}C]



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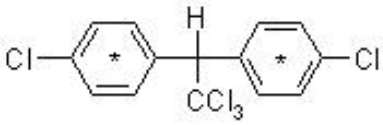

p,p'DDT, [ring-U-^{14}C]

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630 **Figure 1**. Structural formula indicating labeled positions of test compounds

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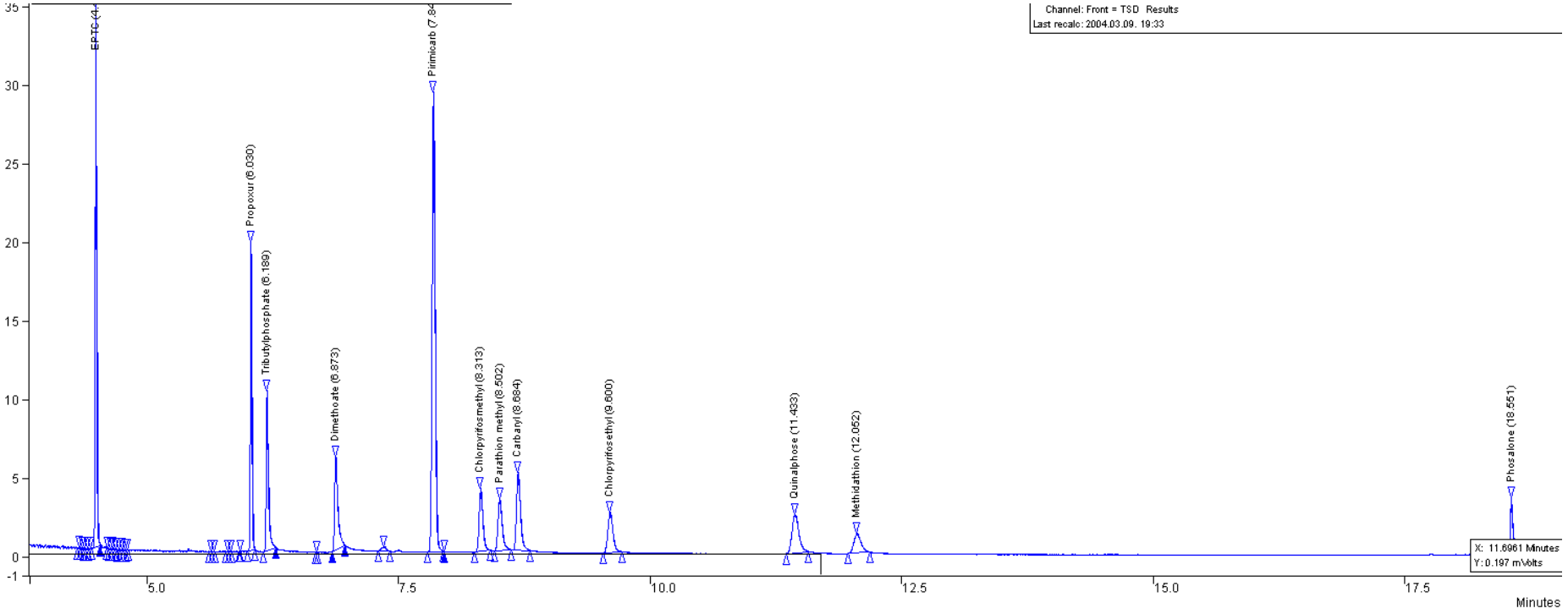
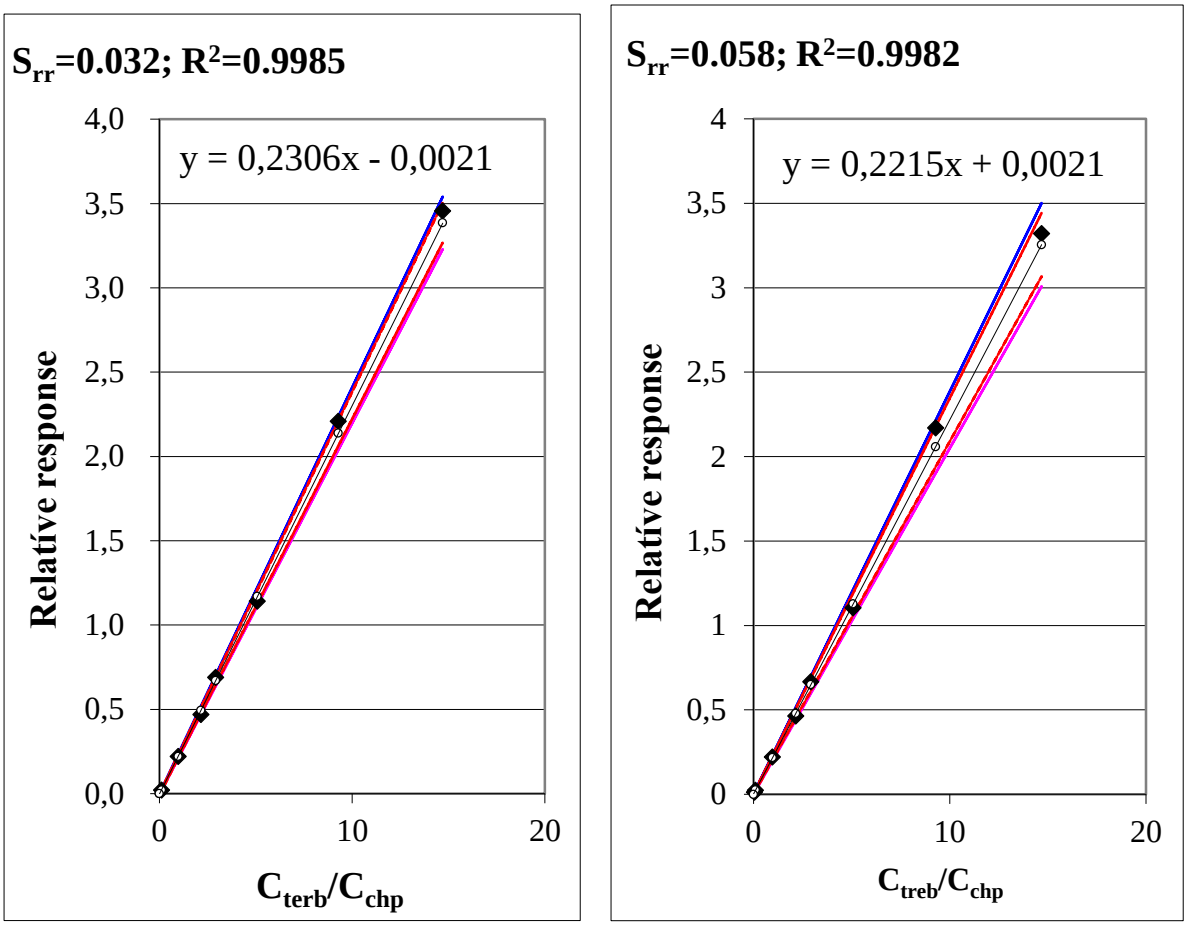


Figure 2 typical chromatogram of the system suitability test mixture containing EPTC, propoxur, tributyl-phosphate, dimethoate, pirimicarb, chlorpyrifos-methyl, parathion-methyl, carbaryl, chlorpyrifos, chinalphos, methidathion and phosalone.



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640 **Figure 3**. Calibration charts of terbutryn on different days. The blue and red lines indicate the

641 confidence and tolerance limits around the weighted regression line. Note the difference in R^2

642 and S_{rr}.

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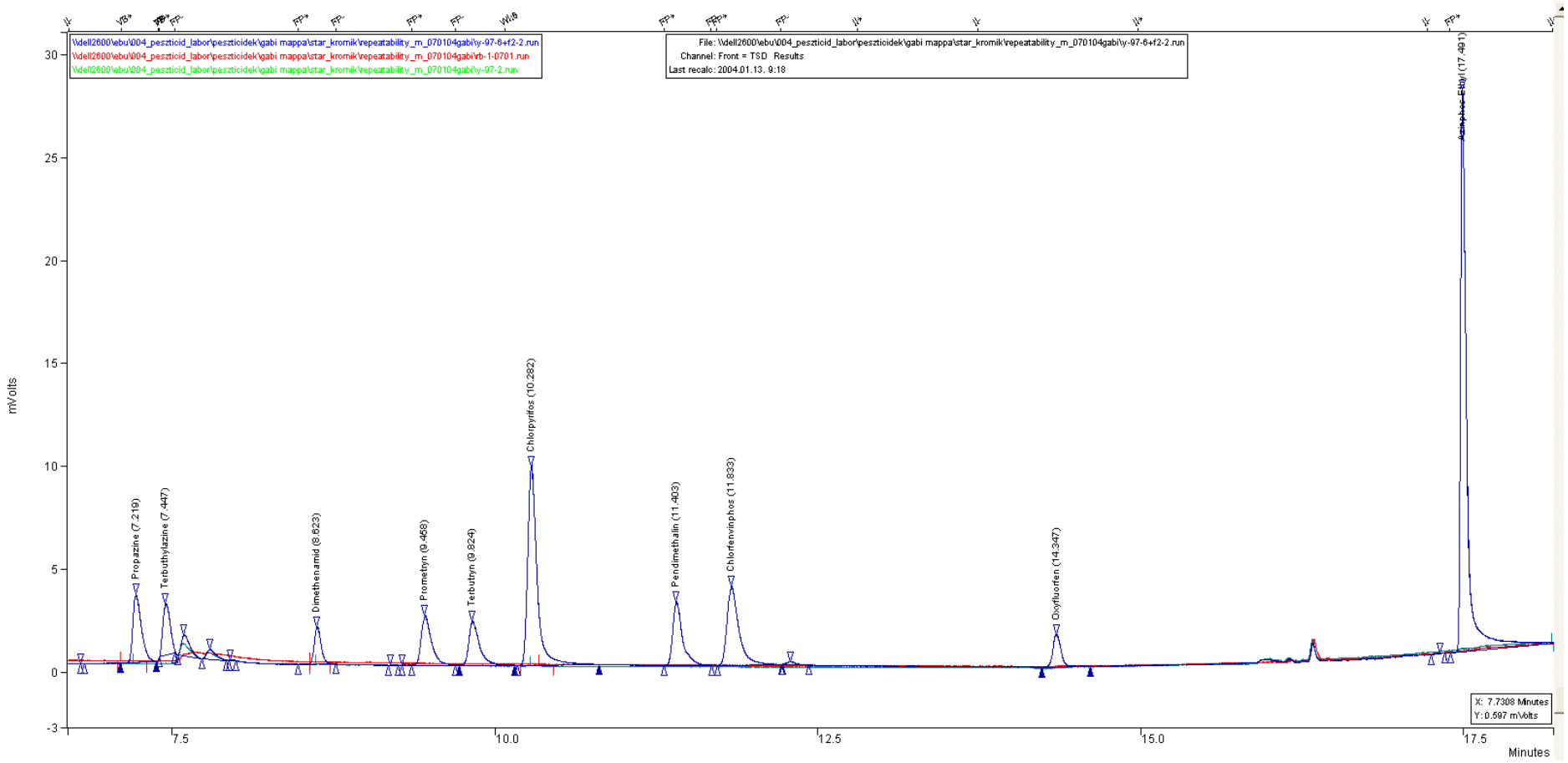

Figure 4. Example for specificity of detection of test compounds in extracts of Y97 soil (red colour), spiked at 20 LOQ level (Blue colour), and reagent blank (green colour).

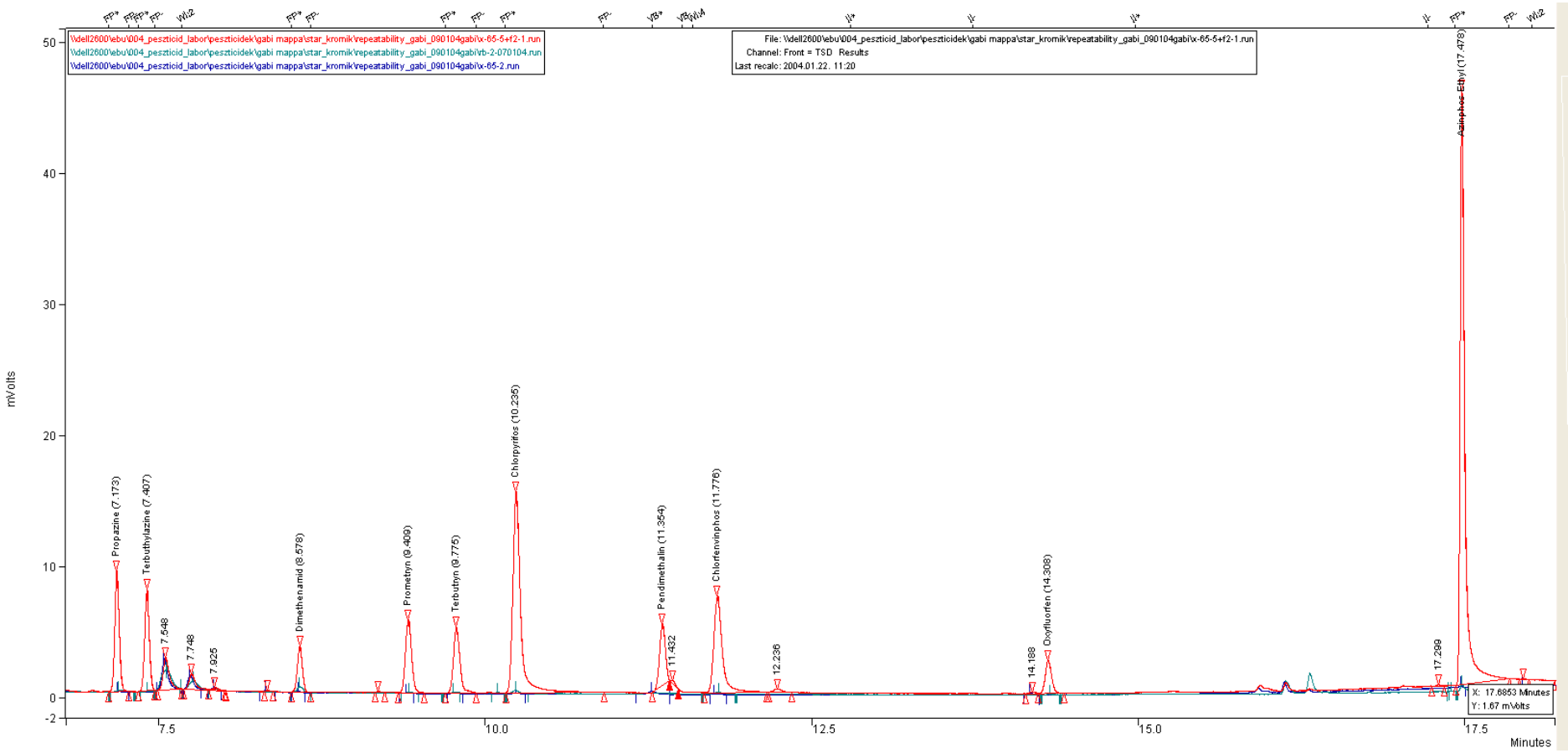

Figure 5. Example for specificity of detection of test compounds in extracts of X65 soil spiked at 20 LOQ level (red colour), bank extract (green colour), blank soil extract (blue colour).

653 **Table 1.** Physical properties of labeled compounds

Name	Water solubility mg/L (20-25 ^{o}C)	Vapour pressure mPa (25 ^{o}C)	Henry constant Pa m^3mol^{-1}	log K_{OW}
Atrazine, (riazol ring ^{14}C)	33	3.85×10-2	1.5×10^{-4}	2.5
Carbofuran, [(2,2-dimetil ,3)-^{14}C]	320	0.031 ×10-2	2.4×10^{-5}	1.52
Chlorfenvinphos, [etil-1-^{14}C]	121	1.0	NA	3.85
Chlorpyrifos [etil-1-^{14}C]	1.4	2.7	0.6761	4.7
p,p'DDT, [ring-U-^{14}C]	0.0055	0.025*	NA	6.91

654 * measured at 20 ^{o}C; NA: not available

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Table 2. Summary of soil parameters

Site, code	dm [%]	Organic matter%	pH	Sand %	Silt %	Clay %
Hercegkút,Y97	86.2	3.14	6.41	33.8	41.6	24.6
Mezőkövesd X65	88.4	2.4	6.8	36.0	26.5	37.5
Olaszliszka, V02	94.0	1.89	6.34	26.3	26.7	46.9
Olaszliszka, U129	92.9	2.09	6.37	37.1	36	28
Hejőkeresztúr, V01	95.7	3.5	6.74	58.2	23.1	18.8
Velm, W33	85.0	3.6	7.69	43	27.5	29.4

The measurements were carried at the Soil Testing Laboratory of Agricultural Service Institute of Fejér County, Hungary

dm: dry matter content

663 **Table 3 .** Performance characteristics of GC determination of test compounds

	Varian GC		Aglient 7890		Varian GC	
Compound	RT [min]	RRT	RT	RRT	LOD pg	LOQ [mg/kg]
Azinphos-ethyl	17.85	1.69	9.913	0.928	5	0.01
Dimethenamid	8.87	0.84	9.154	0.857	20	0.02
Chlorfenvinphos	12.12	1.15	10.52	0.985	10	0.01
Chlorpyrifos	10.56	1.00	10.68	1.000	5	0.01
Oxyfluorfen	14.66	1.39	8.858	0.829	50	0.05
Pendimethalin	11.68	1.11	9.966	0.933	20	0.02
Promertyn	9.71	0.92	9.25	0.886	10	0.01
Propazine	7.43	0.70	10.21	0.955	10	0.01
Terbuthylazine	7.67	0.73	10.14	0.950	10	0.01
Terbutryn	10.09	0.96	11.15	1.044	10	0.01

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667 **Table 4).** Example for the calculation of reproducibility of extraction

Soil type	Activity of extracts (dpm)			A_{spike}	Q	CV_{eQ}
W33/A	7 942.8	7 811.3	7 946.4	7 529.4	1.049	
W33/B	8 057.9	8 080.6	8 016.6	7 594.7	1.060	
W33/C	8 410.9	8 491.3	8 515.7	7 661.0	1.106	
W33/D	7 913.0	7 966.3	7 976.6	7 387.6	1.076	
W33/E	8 205.3	8 166.4	8 199.6	7 683.1	1.066	
				Average Q	1.072	0.020

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672 **Table 5**. Reproducibility of extraction of soil samples

	n	$\bar{Q}_{rec}$[a]	CV$_e$673
Y97	5	0.996	0.085
X65	4	1.101	0.005
W33	5	1.072	0.020
V01	5	0.998	0.005
V02	4	1.045	0.004
U129	5	0.907	0.019
Grand average		1.009	0.0054[b]

674 [a]: average recovery of ^{14}C atrazine after extraction with acetone

675 [b]: calculated from pooled variances excluding two outlier values of 29

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678 **Table 6.** Efficiency of extraction of ^{14}C-labeled test compounds from soil

		Percentage recovery of residues[a]			
		Carbofuran	Chlorpyrifos	Chlorfenvinphos	DDT
X65	Q_E	86.60	73.80	69.00	90.00
	Q_S	13.00	28.50	32.60	9.70
	Q_T	99.6	102.3	101.6	99.7
V01	Q^E	76.38	93.40	85.58	75.73
	Q_S	21.92	8.20	12.72	24.57
	QT	98.3	101.6	98.3	100.3
V02	QE	76.27	83.10	82.84	64.44
	QS	25.8	16.2	15.1	34.8
	QT	102.1	99.3	97.9	99.2

679 [a]: calculated for dry soil as an average of results of 3 replicate tests;

680 Q_E: average ^{14}C activity expressed as parent residue found in the Ac-EtAc combined

681 extract;

682 Q_s: average ^{14}C activity expressed as parent residue remaining in the soil after extraction

683 with Ac-EtAc solvent system;

684 QT: average total ^{14}C recovered activity expressed as parent residue.

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690 **Table 7.** Reproducibility[a] of optimized method applied for soils (X65, Y97, W33)

Spike	Level 1 (LOQ)		Level 2 (20LOQ		Level 3 (100LOQ)	
Compound	$\bar{Q}_{L1}$	CV_Q	$\bar{Q}_{L2}$	CV_Q	$\bar{Q}_{L3}$	CV_Q
Azinphos Ethyl	91.2	0.15	87.8	0.13	81.4	0.10
Chlorfenvinphos	99.9	0.12	79.4	0.13	89.8	0.11
Chlorpyrifos	86.6	0.15	80.5	0.12	83.1	0.09
Dimethenamid	102.7	0.20	75.9	0.10	92.6	0.11
Oxyfluorfen	84.5	0.13	76.3	0.10	88.5	0.11
Pendimethalin	110.8	0.11	75.8	0.11	83.0	0.13
Prometryn	100.9	0.20	87.0	0.18	100.5	0.13
Propazine	96.7	0.15	77.4	0.14	87.1	0.11
Terbuthylazine	110.0	0.15	106.6	0.18	110.7	0.21
Terbutryn	113.7	0.17	85.8	0.16	102.7	0.12

691 [a]: reproducibility was determined from the results of 15 tests performed by 3 analysts on
692 different days
693 $\bar{Q}_{L1}$: average recovery obtained from reproducibility study;
694 CV_Q: relative standard deviation of recovery values
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697 SUPPLEMENTARY INFORMATION

698

699 **Table S1.** Test compounds used for method validation

Compound	Water solubility (mg/l) (20-25 °C)	Vapour pressure mPa (25 °C)	Henry constant Pa m^3mol^{-1}	log K_{OW}
Azinphos-ethyl	4-5	0.32*	2.5×10^{-2}	3.18
Dimetenamid	1200	36.7	8.32×10^{-3}	2.15
Chlorfenvinphos	121 (Z isomer) 7.3 (E isomer)	1.0	NA	3.85 (Z) 4.22 (E)
Chlorpyrifos	1.5	2.7	6.76×10^{-1}	4.7
Oxyfluorfen	0.116	0.0267	8.33×10^{-2}	4.47
Pendimethalin	0.33	1.94	2.728	5.2
Promertyn	33	0.165	1.2×10^{-3}	3.1
Propazine	5.0	0.0039*	1.97×10^{-4}	3.01
Terbuthylazine	9	0.09	2.3×10^{-3}	3.4
Terbutryn	22	0.225	1.5×10^{-3}	3.65

700 * measured at 20 °C; NA: not available;

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Table S2. Operation conditions of gas chromatographs

	Varian 3800	Aglient 6890A
Detector:	TSD 300 ºC	NPD 320 ºC
Injector:	PTV Mod.1079 / high performance liner 280 ºC	Split/Splitless in splitless mode
Column:	CP-Sil-8CB Low Bleed MS (Varian) 25 m * 0.32 mm * 0.25 μm,	HP5UI 30 m- 0.25 μm
Retention gap	2.5 m * 0.32 mm methyl deactivated	2.5 m * 0.32 mm methyl deactivated
Temperature program:	Start: 60 ºC, 0 min raise 1: 25 ºC/min to 160 ºC raise 2: 4 ºC/min to 200 ºC raise 3: 20 /min to 270 ºC, hold for 3.4 min Total run time: 21 min	80 ºC 1 min 32.7 ºC/min to 170 ºC 0 min 10 ºC/min to 310 ºC 1+5 min 19 min
Carrier gas:	He, 4 mL/min	He 1.2 mL/min
Gas supply for detector:	Make up: N_2, 26 mL/min air: 175 mL/min hydrogen: 4.3 mL/min	Make up: N_2 60 mL air: 34 mL/min hydrogen: 3 mL/min

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