1   **Testing the efficiency of extraction of incurred residues from soil with optimized multi-**

2   **residue method**

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9

10   **Abstract**

11   ~~The efficient extraction of pesticide residues from various matrices is of primary importance~~

12   ~~for obtaining unbiased results.~~ The reproducibility of extraction of residues from spiked soil

13   samples and from soils containing incurred residues was tested with ^{14}C-labeled test

14   compounds of different physical-chemical properties. Nearly 100% of the compounds added

15   to the sample before extraction could be recovered with an average reproducibility relative

16   standard deviation (CV) of 5.4%. The additional steps of the determination process (cleanup,

17   evaporation, etc.) contributed to the major part of the variability of the results (CV=10-20%).

18   The incurred residues were most efficiently extracted with acetone for 30 min followed by the

19   mixture of acetone/ethyl acetate 1:1 for additional 30 min. However, they could only be

20   recovered at various extent (64-90% of total residues), underlying the importance of testing

21   the efficiency of extraction. The residues were identified and quantified by gas

22   chromatography applying thermionic detector. The performance parameters of the method

23   complied with the international method validation guidelines, and they proved to be robust

24   and suitable for determination of pesticide residues in soils of widely different physical-

25   chemical properties.

26

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

28   residues.

29

30 **Introduction**

31

32 There are several extraction methods for determining pesticide residues in soil. Traditionally

33 the Soxhlet [1, 2] and the solid phase (SPE) extractions [3-6] are used as official methods in

34 many countries. Their main drawbacks are requiring large volume of solvents, and the lengthy

35 extraction time. Among the new techniques, the supercritical fluid extraction (SFE), [2,7, 8]

36 pressurized solvent extraction (PLE), [9, 10] accelerated solvent extraction (ASE), [11, 12]

37 microwave-assisted solvent extraction (MASE, MAE) [12-17] are the most widely used methods

38 for extraction of pesticides from environmental samples. These methods produce high

39 recovery of residues applying specific expensive instruments and large solvent volumes in

40 some cases. Ultrasonic solvent extractions (USE) is one of the preferred techniques [12,18-22] as

41 it can be performed with less solvents and shorter time. The solid phase micro extraction

42 (SPME) [6, 17, 23] is mainly used for determining volatile compounds.

43

44 Wide range of solvents have been used depending on the purpose of the analysis. Acetone or

45 acetone – water mixture is frequently used [18, 20, 23-25] in which the soil clods fall apart

46 facilitating the complete partition of compounds between the soil and solvent phase.

47 Disintegration of soil particles is further assisted by adding ammonium chloride (NH_4Cl),

48 ammonium phosphate $(NH_4)_3PO_4$.[26-28] The mixtures of acetone with hexane, ethyl acetate

49 (EtAc) or toluene improve the recovery of compounds of wide polarity range. [1, 9, 16]

50 Previously dichloromethane was also used, [24, 25] but currently its use is restricted for

51 protecting the environment. For its replacement EtAc and cyclohexane are applied. [12, 19, 20, 29]

52 The presently applied methods are the variants of those used for residue analysis in food

53 matrices, such as the QuEChERS method [12, 28, 30, 31] involving acetonitril for extraction. The

54   EN 12393-2:2014 Standard, [32] based on acetone or EtAc/cxyclohexane 1:1 solvent

55   extraction, was also applied for soil matrices.[12]

56

57   The methods should be validated before use to provide evidence that they fit for the intended

58   purposes. [33, 34] The generally acceptable main performance parameters are: specificity: signal

59   resulted from untreated control sample is less than 30% of limit of quantification (LOQ)

60   which is the lowest concentration that can be quantified reproducibly with known uncertainty;

61   [35] sensitivity (LOD): typically 0.2 [36] - 0.3 LOQ set generally at 10 times the noise level; [37]

62   matrix effect <±20% compared to response of pure standard solution; the mean recovery:

63   within 70-120%. [34] The linearity and goodness of calibration should be tested with minimum

64   5-point calibration covering the analytical range. Its measure is the standard deviation of

65   relative residuals [38] (S_{rr}) instead of the usually applied coefficient of regression (R^2).

66

67
$$S_{rr} = \sqrt{\frac{\sum(Y_{rel,i} - \bar{Y}_{rel})}{n-2}}$$
(1)

68

69
$$Y_{rel}\% = 100 \times \frac{Y_i - \hat{Y}}{\hat{Y}}$$
(2)

70   Where Y_i is the response and $\hat{Y}_i$ is read from calibration line for x_i calibration concentration, n

71   is the number of ≥ 5 calibration points. Since the standard deviation of the residuals is usually

72   proportional to the injected analyte, the standard deviation of the relative residuals reflects the

73   average variability of the calibration points. Applying weighted linear regression S_{rr} should be

74    $\leq 20\%$. [34]

75   The uncertainty of the measured residue values should be $\leq 20\%$. It is usually expressed as the

76   relative standard deviation obtained from repeatability and within laboratory reproducibility

77   determined from minimum 5 recovery studies.

4

78   Though the Codex Method Validation GLs [33] and the FAO/WHO Joint Meeting on Pesticide

79   Residues (JMPR) [39] list the efficiency of extraction and homogeneity of analytical sample

80   obtained from the laboratory sample as a basic performance characteristics to be tested, the

81   published validation reports rarely include these important parameters. It should be

82   emphasized that neither the analyses of proficiency tests and collaborative study samples nor

83   recovery studies performed with spiked test portions removed from the analytical sample

84   provide information on the homogeneity of analytical sample. The efficiency of extraction can

85   only be determined from these studies, if the samples contain incurred residues.

86

87   The objectives of our study are to test the applicability of widely used solvents (acetone, ethyl

88   acetate and hexane) that can be used with GC-NPD (nitrogen phosphor selective detector) and

89   ECD if GC-MS/MS or LC-MS/MS systems are not available for determination of pesticide

90   residues, optimize the extraction procedure and assess its efficiency for extraction of ^{14}C-

91   labeled incurred residues from soil.

92

93   **Materials and methods**

94

95   *Equipment*

96

97   In addition to the usual laboratory glassware and devices, the following major equipment was

98   used: Beckman 6000 TA liquid scintillation counter (LSC) with automatic quenching

99   compensation; OX400 Biological Oxidizer; Stephan UM 5 Universal and Tecator 2096

100   laboratory homogenizers; Sigma 4K15 centrifuge; Mettler top load (0.01 g) and analytical

101   (0.00001 g) balances; Edmund Bühler SM 25 and Certomat SII sieve shakers; TurboVap

102   (Zymark) solvent evaporator; Varian 3800 gas chromatograph equipped with thermoionic

103   (TSD) detector and PTV injector (1079); CP-Sil-8CB Low Bleed MS column (25m ×

104   0.32mm, df = 0.25μm); and 2.5m × 0.32mm methyl deactivated retention gap. Aglient GC

105   with split/splitless injector and nitrogen, phosphor sensitive detector.

106

107   *Materials*

108

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

110   Calibration Standard for LSC: normal activity of ^{14}Carbon standard (code CRF 101,

111   Amersham International plc, UK) is 5000 disintegrations per minute (dpm). ^{14}C radionuclide

112   purity >99.9%. Half-life 5730 ± 40 years.

113   Absorption Solution for $^{14}CO_2$ for biological oxidizer: 10 mL of ethanolamine/methanol

114   (12.5/87.5, v/v).

115   Filter paper to determine blank background activity of biological oxidizer.

116   Anhydrous calcium chloride (Merck reag. grade).

117   Analytical reference standards >98% purity (Dr. Ehrenstorfer GmbH): azinphos-ethyl (S-

118   (3,4-dihydro-4-oxobenzo[d]-[1,2,3]-triazin-3-ylmethyl) O,O-diethylphosphorodithioate),

119   chlorfenvinphos (2-chloro-1-(2,4-dichlorophenyl)ethenyl phosphate), chlorpyrifos (O,O-

120   diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate), dimethenamid ((RS)-2-chloro-N-(2,4-

121   dimethyl-3thienyl)-N-(2-methoxy-1-methylethyl)acetamide), oxyfluorfen (2-chloro-α,α,α-

122   trifluoro-p-tolyl 3-ethoxy-4-nitrophenyl ether), pendimethalin (N-(1-ethylpropyl)-2,6-

123   dinitro-3,4-xylidine), promertyn (N^2,N^4-diisopropyl-6-methythio-1,3,5-triazine-2,4-diamine),

124   propazine (6-chloro- N^2,N^4-diisopropyl-1,3,5- triazine-2,4-diamine), terbuthylazine (N^2-tert-

125   butyl-6-chloro-N^4-ethyl-1,3,5-triazine-2,4-diamine), terbutryn (N^2-tert-butyl-N^4-ethyl-6-

126   methylthio-1,3,5-2,4-diamine).

127 ^{14}C-labeled reference standards: triazol-ring- ^{14}C-atrazine (6-chloro-N^2-ethyl-N^4-isopropyl-

128 1,3,5-triazine-2,4-diamine) (specific activity 1.6 Mbq/mg radioactive purity 96.5%, provided

129 by Syngenta, 96,5%), (2,2-dimethyl, 3)-^{14}C-carbofuran (CA) (2,3-dihyro-2,2-

130 dimethylbenzofuran-7-yl methylcarbamate), ethyl-1-^{14}C-chlorfenvinphos (CF), Ethyl-1- [14]C-

131 chlorpyrifos (CP) and ethyl-1-^{14}C-p,p'-DDT (DT) (1,1,1-trichloro-2,2-bis(4-

132 chlorophenyl)ethane) >95% radioactive purity provided by the International Atomic Energy

133 Agency (IAEA).

134 The characteristic physical properties and chemical structural formula indicating the label

135 position(s) are summarized in Table 1 and shown in Figure 1, respectively.

136

137 ***Soils used in the experiments***

138

139 About 20 kg soil was collected from the top 15 cm layer at six sites having different physical

140 characteristics (Table 2.) which could affect the recovery of the pesticide residues.

141 The samples were prepared following the ISO 11464:2006 Standard[40] and processed as

142 described by Suszter et al. [41] The two terms are synonyms, but in pesticide residue analysis

143 they indicate different operations.

144

145 Sample preparation: the procedure used, if required, to convert the laboratory sample into

146 the analytical sample, by removal of parts (soil, stones, bones, etc.) not to be included in the

147 analysis. [33]

148 Sample processing: the procedure(s) (e.g. cutting, grinding, mixing) used to make the

149 analytical sample acceptably homogeneous with respect to the analyte distribution, prior to

150 removal of the analytical portion. The processing element of preparation must be designed to

151 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 $$dm = 100 - 100 \times \frac{a-b}{a-c} \tag{3}$$

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 $$Efficiency = \frac{dpm\ standard\ after\ combustion - dpm\ blank\ after\ combustion}{dpm\ standard} \tag{4}$$

176

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

178 The ^{14}C activities of the reference standard, the background activity of soil, as well as the

179 treated soils (500 mg) were determined following the same procedure.

180 For the determination of the ^{14}C activity of the extracts, 12 mL scintillation cocktail and 5 mL

181 extract were transferred into 20 mL LSC vial. The vial was tightly closed, shaken and placed

182 in the Beckman LSC counter.

183 Each sample was measured three times for 5 minutes, after running the self-calibration

184 program, and their average activity was used for further calculations. The average relative

185 standard deviation of the replicate LSC measurements of 29 test portions was 0.0073.

186

187 *Determination of the reproducibility of extraction of spiked samples*

188

189 Six soils of different physical-chemical properties (U129, V01, V02, W33, X65, and Y97)

190 shown in Table 3 were used for studying the reproducibility of extraction.

191 For the treatment of soil test portions, an acetone stock solution containing ^{14}C-labeled

192 atrazine and cold atrazine analytical standard at 0.05 mg/mL concentration with 50,000

193 Bq/mL (3,000,000 dpm/mL) target specific activity was prepared.

194 Test portions of 20 g of processed soil samples were weighed into Petri dish. One thousand

195 μL of 0.001mg/mL atrazine standard solution, prepared from the stock solution, was spread

196 over the soil surface with Hamilton syringe (spike level 0.05 mg/kg). The spiked sample was

197 kept in fume hood for 30 min to evaporate the acetone, then transferred into a 250 mL

198 centrifuge tube and 2.8 mL of 0.2 mol NH_4Cl solution and 40 mL acetone were added. The

199 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
$$Q = \frac{A_E - A_0}{A_{spike}} \qquad (5)$$

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

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

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

225   three replicates.

226   Twenty grams of processed soils were weighed in 250 mL round bottom flask and treated

227   with 30 mL acetone containing the standard solution at 0.05 mg/kg dry soil equivalent. Each

228   standard solution was applied to different portions of soil. The flask was fixed on rotary

229   evaporator and rotated for 15 minutes at ambient temperature to thoroughly mix the soil and

230   the solvent, then the solvent was evaporated under gentle vacuum immersing the flask into

231   water bath kept at 35°C. The dry, free flowing soil powder was transferred to 100 mL

232   centrifuge tube with screw cap. Distilled water was added until water holding capacity of the

233   soil and the container was stored in the greenhouse of the IAEA at about 25°C for 6 months.

234   The evaporated water was replaced regularly. Twelve replicates were prepared from each of

235   the soil-pesticide combinations. Untreated soils were processed similarly to fortified ones and

236   they were used to determine the background activity. They also served as blank sample for

237   validation of the optimized method.

238   The exact initial ^{14}C activities of the fortified soils containing the incurred residues and the

239   blank soils were determined just before their extraction, as described above.

240

241   After 6 months of storage, the soil samples were extracted with either of hexane:acetone (1:1

242   v/v), acetone and ethyl acetate (EtAc). The extracting solvents were selected from those

243   which have been most frequently used for determination pesticide residues in soil and plant

244   materials. Dichloromethane was not considered in view of protection of the environment. The

245   three solvents have high and medium polarity and non-polar character. They were primarily

246   suitable for extraction of pesticides of similar polarity.

247

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

11

249 1.   Before extraction, 2.8 mL 0.2 mol NH_4Cl was thoroughly mixed with the 20 g soil

250 sample and let to stand for 15 minutes, then 40 mL of one of the extraction solvents was

251 added, the container was tightly closed and agitated with horizontal shaker at 200-250 rpm for

252 30 min.

253 2.   The tube was centrifuged at 3000 rpm, 1-1 mL of clean extract were withdrawn and

254 mixed with 12-12 mL scintillation cocktail in LSC vials. The radioactivity was determined for

255 3×5 minutes with Backman LSC counter.

256 3.   The tube was agitated again with horizontal shaker, for another 30 mins (total

257 extraction time 1 hr)

258 4.   Step 2 was repeated and the extraction was continued for another hour (total extraction

259 time 2 hours).

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

261

262 Based on the results of the first series of tests, an additional extraction procedure was tested:

263 the 20 g soil was first extracted with 20 mL acetone, then 20 mL ethyl-acetate was added and

264 the agitation of the soil was continued for 30 mins (total extraction time 1 hour). The use of

265 combination of solvents was necessary, because acetone completely disintegrated the soil

266 particles which increased the efficiency of extraction and the ethyl acetate extracted non-polar

267 residues as well.

268 The extracting solvent was decanted after the end of the extraction. The soil was rinsed with

269 20 mL extracting solvent, centrifuged, the supernant solvent was decanted and the soil was

270 kept under fume hood until constant weight was reached. The radioactivity of the extracted

271 soil was determined from 500 mg portions.

272 All measured residue concentrations were expressed on dry soil basis.

273

274 *Description of optimized analytical procedure*

275

276 Azinphos-ethyl, dimethenamid, chlorfenvinphos, chlorpyrifos, oxyfluorfen, pendimethalin,

277 promertyn, propazine, terbuthylazine and terbutryn were selected as test compounds

278 representing wide range of water solubility, volatility and octanol – water partition coefficient

279 (supplementary information Table S1) like those of ^{14}C-labeled test compounds used for

280 studying the efficiency of extraction (Table 1).

281 The untreated soils were spiked with the mixtures of standard solutions at concentration levels

282 equivalent to LOQ, 20LOQ and 100LOQ. Three different types of soil samples (X65, V01

283 and V02) were processed with adding sufficient water as described by Suszter et al. [41]

284 Twenty grams of processed soil was weighed into centrifuge tube, 2.8 mL 0.2 mol NH$_4$Cl was

285 added and mixed with the soil with a glass road. Twenty mL acetone containing 120 ng

286 chlorpyrifos/mL internal standard (ISTD) was added, the tube was tightly closed and agitated

287 on a horizontal shaker at 200 rpm for 30 minutes. Twenty mL ethyl acetate was added to the

288 extract and the shaking was continued for 30 minutes. The soil was let to settle and the tube

289 was centrifuged at 3000 rpm. Ten mL extract (equivalent to 5 g soil) was pipetted into 20 mL

290 test tube, it was dried by shaking with 6 ± 0.1 g anhydrous sodium sulfate for 20 seconds, and

291 transferred into a 20-mL calibrated glass test tube through filter paper inserted in a glass

292 funnel. The centrifuge tube and the filter funnel was rinsed with 3×2 mL EtAc. The solvent

293 was evaporated with nitrogen to about 0.5 mL with TurboVap®VL evaporator at maximum

294 30 ºC and 1 psi pressure. The final volume was adjusted exactly to 2 mL. No further cleanup

295 was employed.

296

297 The qualitative and quantitative determination of the residues was carried out with Varian

298 3800 GC equipped with nitrogen and phosphorus selective thermionic detector (TSD) and

13

299   PTV injector. Aglient 7890A GC with NPD was used for confirmation of the identity of the

300   analytes. The chromatographic conditions are summarized in supplementary information

301   (Table S2). The condition of the chromatographic system (resolution, phosphorus-carbon and

302   nitrogen-carbon selectivity, peak asymmetry, stability of retention times) was checked by

303   injecting the system suitability test mixture [38] at the beginning and at the end of each batch of

304   chromatographic analyses of sample extracts. An example chromatogram of the SST mixture

305   is shown in Figure 2. If the system suitability test indicated malfunction the appropriate

306   maintenance actions were taken.

307   The matrix effect was compensated by preparing the calibration standard solutions from the

308   blank soil extracts. The concentration of the test compounds in the calibrating standard

309   solutions ranged from 0.5 LOQ to 150 LOQ. The weighted linear regression lines, based on 5

310   concentration points, and their confidence intervals, as well as the S_{rr} values were calculated

311   with a self-made Excel template. Examples for typical calibration charts are given in Figure 3.

312   The LOD, LOQ, RT and RRT are summarized in Table 3.

313

314   The specificity of the detection was checked with injecting the standard mixture, extracts of

315   the soil and reagent blanks. The specificity was acceptable if no interfering peak was larger

316   than 0.3LOQ. Examples for the three chromatographic runs are shown in Figures 4 and 5.

317

318   The compounds were identified based on their retention times relative to chlorpyrifos ISTD

319   (RRT) (Table 3). The ratio of peak areas of analytes and ISTD were evaluated with Star 6.2

320   software for the quantitative determination.

321

322   **Results and discussion**

323

324 ***Reproducibility of extraction***

325

326 It was tested on five different soils with ^{14}C-atrazine at spike level of 0.05 mg/kg. Four or five

327 test portions from each soil was spiked and extracted with acetone on different days by 3

328 analysts. The radioactivity of each extract was measured 3 times for 5 min with Beckman

329 LSC. The recovery of ^{14}C-atrazine was calculated with equation 5 from the average recovered

330 activity and the activity of the spiking solution. The potential outliers were tested with

331 Grubb's and the homogeneity of variances with Cochran tests. [42] The average recoveries

332 obtained from 28 independent recovery tests performed with the 6 different soils were

333 compared with analysis of variances (ANOVA) [42], however none of the neighboring ones, in

334 the rank ordered values, differed more than the least significance difference (0.249) indicating

335 that there was no difference in the recoveries from different soils. Consequently, the grand

336 average of recoveries could be calculated from all recovery data. The calculation of the

337 reproducibility of extraction (CV_{eQ}) is shown with an example in Table 4. The reproducibility

338 of extraction of 5 types of soil samples was calculated by pooling the CV_e values obtained

339 with different soils. The results, indicating complete recovery (100.9%) of all tests with a

340 pooled CV_{extr} (0.0054), are summarized in Table 5.

341

342

343 ***Efficiency of extraction of incurred residues***

344

345 The ^{14}C-activites derived from incurred residues of carbofuran, chlorfenvinphos,

346 chlorpyriphos and p-p-DDT were determined in three different soils (V01, V02 and X65)

347 after 6-month storage at about 25 $^{\circ}$C.

348   In some cases, the recovered ^{14}C activity has remained practically the same after 30 minutes.

349   However, in other cases the recovery improved (DDT–EtAc, chlorpyrifos–acetone,

350   chlorfenvinphos–EtAc) significantly if the shaking was continued for 60 minutes, and

351   remained practically constant afterwards. Acetone completely disintegrated the soil particles,

352   while in case of hexane and EtAc some clods were formed or remained in the extracted soil.

353   During the extraction with acetone-hexane mixture two phases were formed. The upper

354   hexane-acetone phase contained mainly the non-polar compounds, while the polar compounds

355   partitioned into the lower (acetone–water) phase, which is not desirable for quantitative

356   determination of residues.

357   Based on the experience gained with various solvents and extraction time, we concluded that

358   starting the extraction with acetone for 30 mins, adding EtAc and continuing the shaking for

359   another 30 minutes would give the highest recoveries for pesticide residues having wide range

360   of polarity. The optimal proportion of soil extracting solvent was not tested, but taken from

361   many publications applying the soil/solvent ratio of 1:2. Taking into account the vast

362   experience with the application of QuEChERS method [43] acetonitrile would be a generally

363   applicable solvent for extracting residues from soil [28, 30, 31] if MS detection would be used,

364   however acetonitrile cannot be directly used with N-P selective thermoionic and electron

365   capture detectors, therefore its applicability was not tested.

366

367   The remaining activities in the extracted soil was measured after the combined acetone–EtAc

368   extraction procedure. The results, summarized in Table 6, show that the proportion of ^{14}C

369   activity in the soil varied in different pesticide–soil combinations.

370   As the adsorption of pesticide residues to soil particles and their partition between

371   soil–extracting solvent depend on the combination of several physical-chemical properties of

372   soil, the number of tests and combinations did not allow detailed analyses of their

373   relationship. Nevertheless, our experiments clearly indicate the importance of testing the

374   efficiency of extraction with incurred residues as part of the validation or extension of the

375   scope of a method. The most convenient way of testing the efficiency of extraction is to use

376   ^{14}C-labeled test compounds, but they are not readily applicable in routine pesticide residue

377   laboratory. Therefore, the Codex GLs on method validation [33] and the FAO JMPR Manual

378   [39] provide some generally applicable alternative procedures.

379   It is emphasized that the measured activities include the parent compound and its metabolites

380   which contain [14]C. The proportion of parent compound and metabolites depends on several

381   factors such as the time between pesticide application and sampling, microbiological activity,

382   pH and physical properties of soil, therefore the concentration of the parent compound would

383   be lower than that indicated by ^{14}C measurement.

384

385   *Applicability of optimized procedure for analysis of pesticide residues in soil*

386

387   The test mixture of 10 pesticide active substances having wide range of physical-chemical

388   properties (Table S1) were used to spike 3 different types of soils at 3 concentration levels of

389   100-fold difference. The linearity of the response of components of the standard mixture was

390   established in the range of 0.5 LOQ and 150LOQ. The goodness of calibration, was

391   characterized by the coefficient of regression (R^2) and the standard deviation of relative

392   residuals (S_{rr}). Both parameters were well within the acceptable range specified by the

393   European Union Quality control guidance document. [34]

394   The reproducibility of determination of residues from spiked samples was tested with 5

395   replicates in each soil and spike level. The results revealed that there was no difference among

396   the reproducibility of analyses depending on the type of soil, which is in line with the findings

397   of reproducibility of extraction. The average recoveries ($\bar{Q}_{L1}$) and reproducibility relative

17

398   standard deviations (CV_Q), summarized in Table 7, are within the acceptable limits of the

399   corresponding quality control guidelines.

400

401   **CONCLUSIONS**

402

403   Use of ^{14}C-labeled compounds enabled quantifying the analytes present in the LSC cocktail

404   with an average 0.0073 relative uncertainty. Our results proved that the residues can be

405   extracted from spiked soil samples with an average CV_e of 0.54%. The major part of the

406   variability of results of residue analysis derived from the further steps (evaporation, cleanup

407   and instrumental analyses), which may require special attention if the combined relative

408   reproducibility uncertainty of the results is getting close to the upper acceptable limit of 25%.

409   [34] The efficiency of extraction depends on several factors and up to about 35% of total

410   residue might remain unextracted which can lead to biased results. The recovery tests

411   performed with spike samples do not reveal the required information. Therefore, the

412   efficiency of extraction should be tested with incurred residues in every case when a new

413   extraction procedure is validated or an established method is extended to a new matrix. For

414   this purpose, alternative methods [39] are available if the application of ^{14}C-labelled

415   compounds is not feasible.

416

417   In the lack of GS-MS/MS, LC-MS/MS instruments, the GC with specific detectors and

418   appropriate cleanup procedures can be reliably used for determination of pesticide residues

419   especially in samples of know pesticide treatment history, or in selective field surveys

420   targeted for specific pesticide residues.

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422   **ACKNOWLEDGEMENT**

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FIGURE CAPTIONS

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

590   **Figure 2**. Typical chromatogram of the system suitability test mixture containing EPTC,

591   propoxur, tributyl-phosphate, dimethoate, pirimicarb, chlorpyrifos-methyl,

592   parathion-methyl, carbaryl, chlorpyrifos, chinalphos, methidathion and phosalone.

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

594   confidence and tolerance limits around the weighted regression line. Note the

595   difference in R^2 and S_{rr}.

596   **Figure 4.** Example for specificity of detection of test compounds in extracts of Y97 soil (red

597   colour), spiked at 20 LOQ level (Blue colour), and reagent blank green colour)

598   **Figure 5**. Example for specificity of detection of test compounds in extracts of X65 soil

599   spiked at 20 LOQ level (red colour), bank extract (green colour), blank soil extract

600   blue colour

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628   FIGURES

Atrazine [trazol ring-^{14}C]

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

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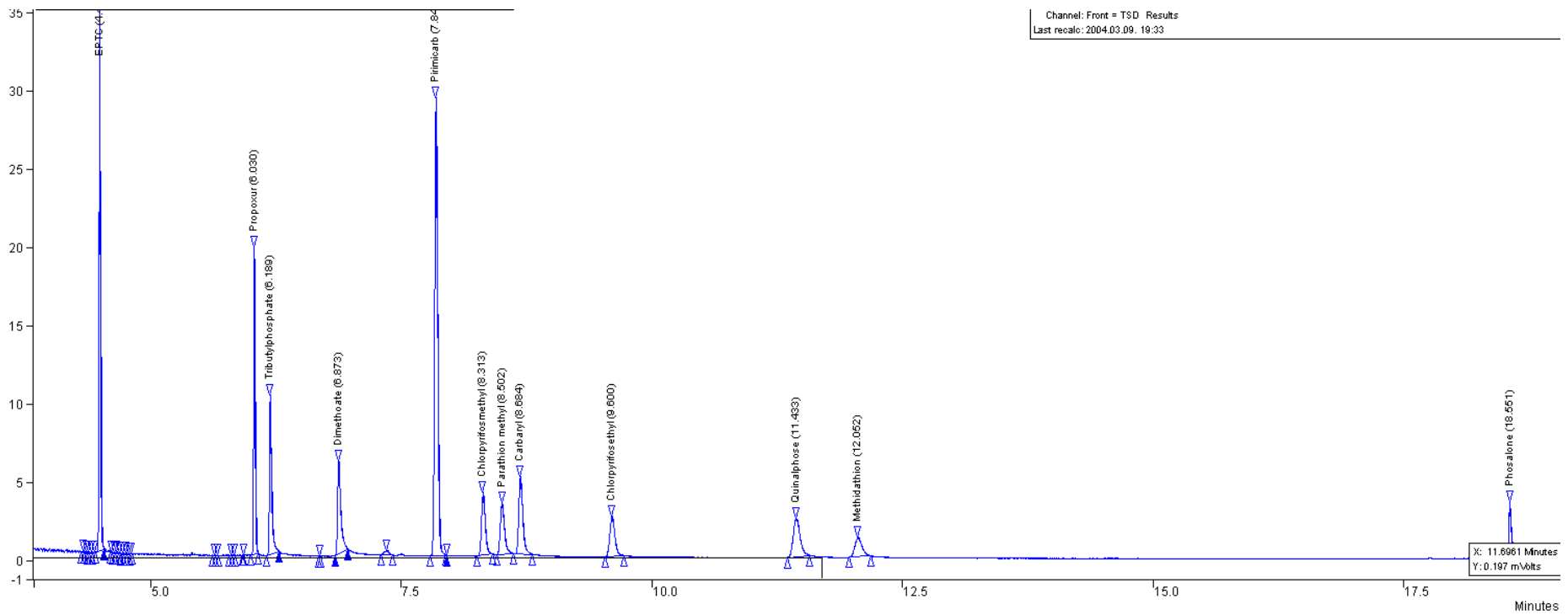
Chlorfenvinphos [ethyl-1-^{14}C]

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

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630   **Figure 1**. Structural formula indicating labeled positions of test compounds
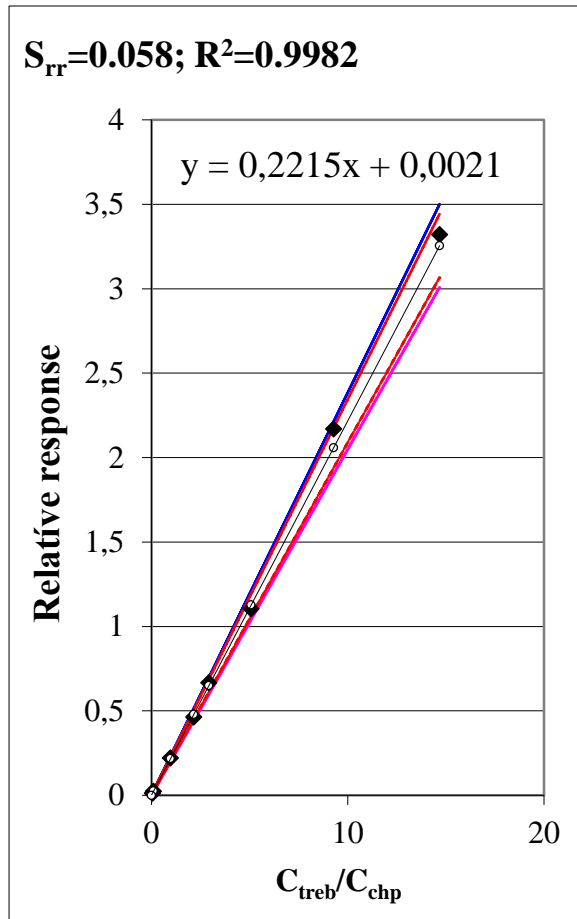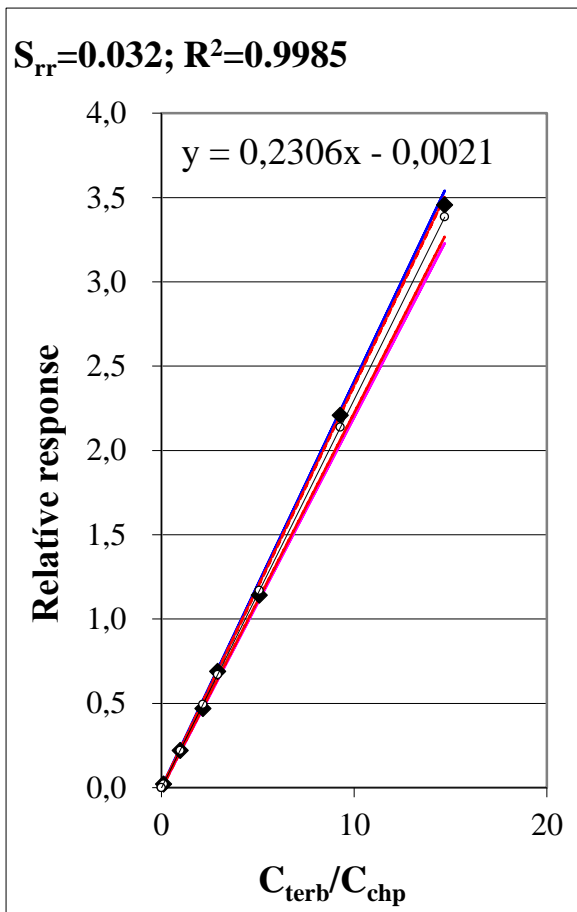
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634   **Figure 2** typical chromatogram of the system suitability test mixture containing EPTC, propoxur, tributyl-phosphate, dimethoate, pirimicarb,

635   chlorpyrifos-methyl, parathion-methyl, carbaryl, chlorpyrifos, chinalphos, methidathion and phosalone.
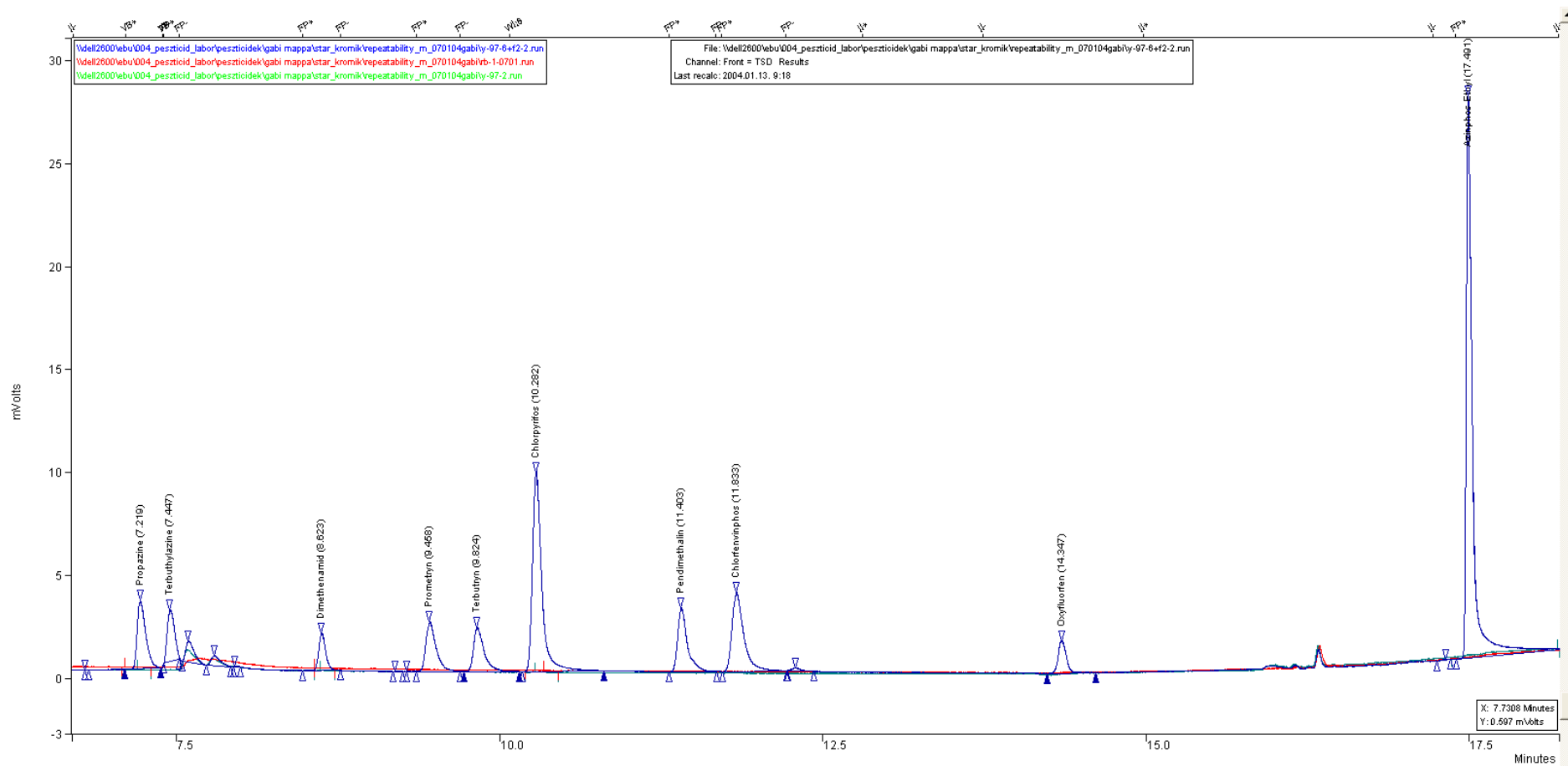
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$S_{rr}=0.032; R^2=0.9985$

$y = 0,2306x - 0,0021$

Relative response

C_{terb}/C_{chp}

$S_{rr}=0.058; R^2=0.9982$

$y = 0,2215x + 0,0021$

Relative response

C_{treb}/C_{chp}

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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
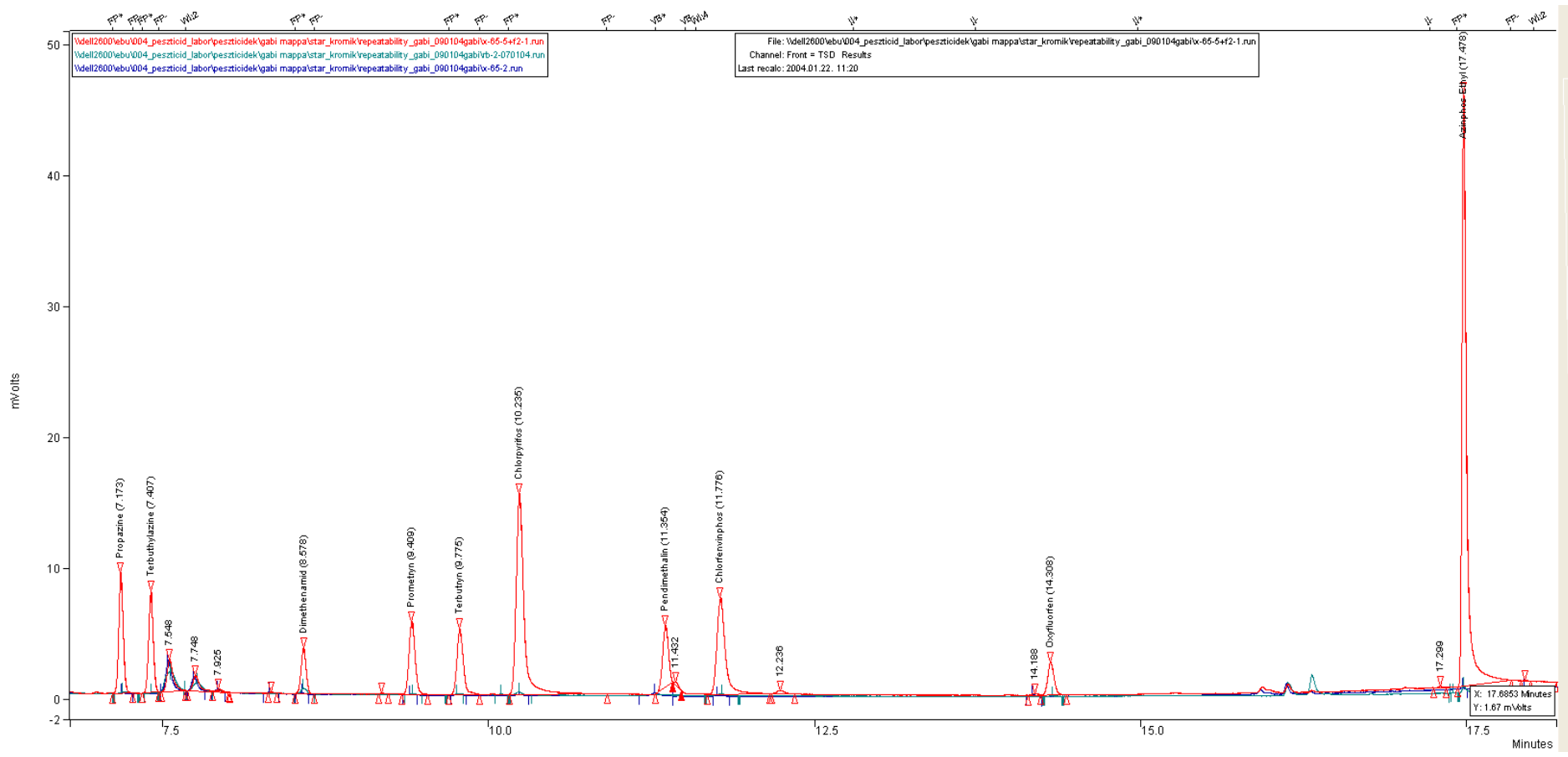
642 and S_{rr}.

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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

647   reagent blank (green colour).

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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).

652

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

Name	Water solubility mg/L (20-25 ºC)	Vapour pressure mPa (25 ºC)	Henry constant Pa m^{3}mol^{-1}	log K$_{OW}$
Atrazine, (riazol ring ^{14}C)	33	$3.85\times10\text{-}2$	1.5×10^{-4}	2.5
Carbofuran, [(2,2-dimetil ,3)-^{14}C]	320	$0.031\times10\text{-}2$	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 ºC; NA: not available

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656 **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

657 The measurements were carried at the Soil Testing Laboratory of Agricultural Service

658 Institute of Fejér County, Hungary

659 dm: dry matter content

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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_e673
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 ºC)	Vapour pressure mPa (25 ºC)	Henry constant Pa m^{3}mol^{-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 ºC; NA: not available;

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702 **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	80 ºC 1 min
	raise 1: 25 ºC/min to 160 ºC	32.7 ºC/min to 170 ºC 0 min
	raise 2: 4 ºC/min to 200 ºC	10 ºC/min to 310 ºC 1+5 min
	raise 3: 20 /min to 270 ºC, hold for	
	3.4 min	19 min
	Total run time: 21 min	
Carrier gas:	He, 4 mL/min	He 1.2 mL/min
Gas supply	Make up: N_2, 26 mL/min	Make up: N_2 60 mL
for detector:	air: 175 mL/min	air: 34 mL/min
	hydrogen: 4.3 mL/min	hydrogen: 3 mL/min

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