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Testing the Stability of Pesticides During Sample Processing for the Chlorpyrifos and Malathion Residue Analysis in Cucumber, Including Matrix Effects

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Abstract This article describes the procedure of the testing the stability of compounds during sample processing for the pesticide residue analysis in cucumber, including matrix matched assessment. To find out significant differences, one-tailed *t* test was applied to the data at 95% confidence level. Our results showed that the calculated value was bigger than the critical value ($t_{calc} > t_{crit}$), which means the pesticides were decomposed under our processing conditions.

Keywords Pesticide stability · Matrix-matched calibration · Sample processing

Sampling, sample processing and analysis may influence the uncertainty of the residue data. The reason of the uncertainty of the sample processing is the losses of compound that can occur during comminution and mixing. Losses of pesticides at this stage and subsequent analytical steps will result in an underestimation of the residue level, with implications for MRL compliance monitoring and consumer risk assessments. Therefore, the stability of residues during sample processing has to be studied in the method validation procedure. The efficiency of sample processing can be improved by applying dry ice to the sample. Stability of pesticides depends on physical and chemical properties of pesticides, sample matrix involved, and the processing condition (Hill et al. 2000; Fussell et al. 2002; Anonymous 2006).

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A number of workers have investigated the stability of pesticides during the processing (Hajslova et al. 1998; El-Bidaoui et al. 2000; Ambrus 2004). A systematic study (Hill et al. 2000) revealed decreases 40%–70% in the concentration of pesticides during sample processing at ambient temperature. Various chemical reactions and evaporation during processing can be responsible for the losses. In a similar work (Fussell et al. 2002), an assessment of the stability of pesticides during the cryogenic sample processing of apples has been undertaken. The result demonstrated that cryogenic processing improved the stability.

The assessment of matrix effect is also important as reported in literature (Gonzalez et al. 2002; Patel et al. 2003; Poole 2007). Matrix-induced effect leads to systematic error in the quantification of the pesticides if a solvent calibration is used. It can be reduced by using effective cleanup methods. Despite the use of such measures, matrix effects can still affect the results. There are two approaches to eliminate matrix-effects: (1) to use matrix-matched calibration standards or analyte protectants, (2) to set correction function and to use this function with the solvent calibration. The most usual and recommended way to avoid such effects is the use of matrix-matched calibration, which corresponds matrix contain in the sample.

This work focused to present calculations for the stability testing of compounds during sample processing including matrix effect, for the pesticide residue analysis.

Materials and Methods

The standard chlorpyrifos-ethyl and malathion, with purities of 99.5%, were obtained from Dr. Ehrenstorfer

Laboratories GmbH, Germany, via IAEA. All other solvents such as, ethylacetate (EtAc), cyclohexane and isooctane were analytical grade from Merck, with the purity of 99.5%, 99.9% and 99.5%, respectively.

A-5 unit of cucumber was cut into half in the longitudinal direction. The units with their cut surface were placed on a tray covered with clean aluminium foil, then 1 mL of stability fortification solution (0.030 mg/mL), including chlorpyrifos and malathion, was applied as surface treatment at the 0.2 mg/kg level (hereafter it will be referred as STB-F level). Sample materials were kept under fume hood for 15 min to allow the pesticide interact with the matrix. The units were placed in the bowl of the blender (Waring Commercial Blender, USA) by avoiding to touch the treated part. To obtain homogenous material, all of the samples were processed with Waring blender at ambient temperature and at high speed for 2 min.

Analytical portions of 30 g sample were withdrawn from blended sample and this was replicated for 7 times. Five grams of $NaHCO_3$ was added to the analytical portion, and mixed. Na_2SO_4 and EtAc were added to the sample at the ratio of 1/1 w/w and 2/1 v/w, respectively, too. The mixture was extracted by using Ultra Turrax (T25 basic Ika-Werke) at 25°C for 30 s for all samples. The extracted material was centrifuged (Beckman Model TJ-6) for 10 min, at 2,500 rpm. The liquid part of the material in the tube was collected, and the volume and weight of extracts recorded.

For the lowest fortification level (0.02 mg/kg) of method validation experiment, 60 µL from 0.01 mg/mL fortification solution (hereafter it will referred as F_1 level) was added to each processed analytical portion just before EtAc addition.

A 1/3 portions of total extract volume from each extract, which corresponds 10 g sample equivalent, was filtered through 60 g Na_2SO_4 in a round bottomed flask. Then the filter cake was washed for three times with 20 mL EtAc, and allowed for the removal of solvent completely. The filtrate was concentrated to 1–2 mL in a rotary evaporator, then concentrated extract was transferred to a calibrated conical test tube in which the evaporation was continued until 1 mL was obtained with gentle N_2 stream. For changing the solvent, 1 mL of the solvent mixture of EtAc/cyclohexane 1/1 v/v was added and finally evaporated to 0.8 mL. The final volume of extract was adjusted exactly to 1 mL for the GPC (semi-automatic KL-SX-3) cleanup (Tiryaki and Aysal 2005).

The GPC column (20 cm × 1 cm glass column) was filled with 10 g Bio-Bead SX-3 (200–400 mesh, Bio-Rad Lab.) gel and calibration of the column was performed with chlorpyrifos and malathion mixture in triplicate. An aliquot of 500 µL from 0.001 mg/mL pesticide mixture containing 500 ng of mixture was injected, then the pesticides were eluted with EtAc/cyclohexane 1/1 v/v at the 0.9 mL/min flow rate. The extracts were cleaned up in the GPC system by injecting 500 µL sample extract (corresponding 5 g of original sample matrix) at flow rate of 0.9 mL/min. According to our determined elution profile, 7–20 mL of pesticide fractions were collected. After changing the solvent, the final volume of combined collected eluent was adjusted with isooctane to 4 mL which corresponded 1.25 g sample equivalent/mL. This procedure was the same for all the samples including fortification and blank. A 2-mL of GPC cleaned extract was evaporated to 0.5 mL and added 1 mL isooctane. After the evaporation final volume was again adjusted to 1 mL (corresponded 2.5 g sample equivalent/mL) in isooctane for GC analysis.

For the GC analysis of cleaned up extracts, HP 6890 GC-NPD were used at the following conditions: capillary column (30 m length × 320 µm id × 0.25 µm nominal film thickness, HP 19091S-433, HP-5MS 5% Phenyl Methyl Siloxane); carrier gas nitrogen 2.0 mL/min, hydrogen 3.0 mL/min; air 60.0 mL/min. Operating Conditions; Column temperature: 70–270°C; initial time 1 min at 70°C; rise (I):20°C/min to 160°C–0 min, rise (II): 4°C/min to 270°C–10 min, total run time: 43 min; detector temperature: 300°C, injector temperature: 200°C (splitless), injection volume: 2 µL. To perform analysis in one calibration curve, at the range of 20–60 pg/µL, fortification samples were diluted to fit the calibration range. Matrix contents after the dilution were 0.25 and 2.5 g sample equivalent/mL, for STB-F and F_1 samples, respectively. Therefore, to compensate for matrix effect for the quantiation of STB-F and F_1, matrix calibration solutions were prepared in blank extract, containing 0.25 g and 2.5 g sample equivalent/mL, respectively. Hereafter they will be referred as matrix calibration/0.25 g seq and matrix calibration/2.5 g seq. GC injections, including solvent calibration2 and both level matrix calibration, were performed in duplicate, except in case of STB-F, where in triplicate. Schematic diagram of all the analytical steps were illustrated in Fig. 1.

Results and Discussion

Calibration curves prepared in solvent were compared with calibration curves prepared in a blank matrix extract (F_0). This helps to establish whether the matrix induces systematic or proportional errors in the quantification of the pesticides. The linearity of all 3 calibration curves were determined by computing r and $S_{\Delta y/\hat{y}}$ value (Huber 2004). The virtue of GC calibration mostly can be characterized by the standard deviation ($S_{\Delta y/\hat{y},\, n-2}$) of the relative residuals (residuals/predicted $\Delta y_i = y_i - \hat{y}$; $Y_i = \Delta y_i/\hat{y}$), which is calculated with n−2 degrees of freedom by using Eq. 1 (Miller and Ambrus 2005).

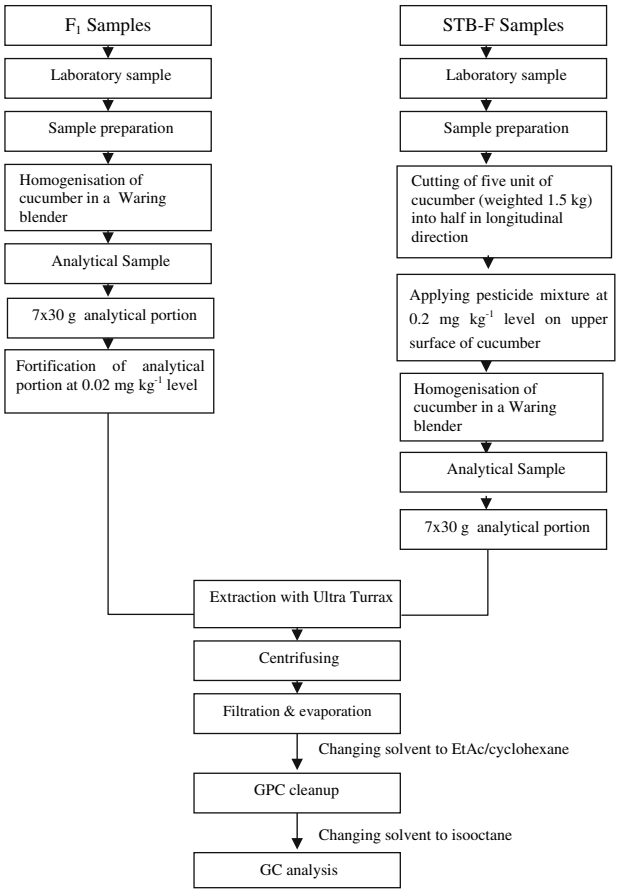

Fig. 1 Overview of analysis used to assess stability of pesticides

$$s_{\Delta y/\hat{y}} = \sqrt{\frac{\sum (Y_i - \bar{Y})^2}{n - 2}} \quad (1)$$

where y_i is the response obtained from injecting analytical standard. $\hat{y_i}$ is the point corresponding with analytical standard on the regression line. $\bar{Y}$ is the mean value of Y_i. n is the total number of standard injections.

The curves of chlorpyrifos and malathion were linear over the range 20–60 pg/μL, with r, $S_{\Delta y/\hat{y}}$ and regression equation. This is given in Table 1, for 3 different calibration. As shown in the table, the calculated $S_{\Delta y/\hat{y}}$ were less than the required limit of 0.1. Calculations of the analyte concentration were performed with weighted linear regression. The straight line determined is referred to as a weighted calibration line: the calculation is more complex, as the weights for the individual points must be included (Miller and Ambrus 2005). An excel template, developed by Agrochemicals Unit of IAEA-Seibersdorf Laboratory, was used for the calculation.

The matrix effect varies depending on the type and concentration of matrix in the extract, condition of chromatographic column and the analyte concentration in the extract, which may vary from day to day (Soboleva et al. 2000). Quantitation of pesticide was performed by using matrix equivalent calibration. Thus, matrix calibration/2.5 g seq and matrix calibration/0.25 g seq were used to quantify the residues in the F_1 and STB-F samples, respectively. By applying calculations and calibrations, the recoveries of chlorpyrifos and malathion from the fortified F_1 and STB-F samples are given in Table 2. The average recovery, S_d and *RSD%* are also included in the table. Table 2 also summarizes the comparison of the average recoveries of compounds, calculated based on solvent calibration and both matrix calibration. The figures showed that the recoveries based on solvent calibration were less than matrix calibration. For instance, the average recovery of chlorpyrifos for F_1 samples in solvent calibration, and corresponded matrix calibration/2.5 g seq calibration were 56.22% and 65.08%, respectively. Similarly, chlorpyrifos average recoveries were 52.80% and 80.91% in solvent

Table 1 Summary of calibration parameters for the compounds for GC-NPD detection with five-level calibration, in solvent and sample matrix

Analyte	Linear range, pg/μL	Calibration and/or analytic function,[a] $y = a+bx$	Correlation coefficient, r	Relative residual standard deviation, $S_{\Delta y/\hat{y}}$
Calibration in solvent				
Chlorpyrifos	20–60	$y = -3.624 + 2.4597x$	0.970	0.059
Malathion	20–60	$y = -4.853 + 1.8229x$	0.962	0.079
Calibration in sample matrix (0.25 g/mL seq)				
Chlorpyrifos	20–60	$y = 1.393 + 1.6978x$	0.971	0.071
Malathion	20–60	$y = 4.799 + 1.2821x$	0.958	0.087
Calibration in sample matrix (2.5 g/mL seq)				
Chlorpyrifos	20–60	$y = -6.968 + 2.2278x$	0.977	0.052
Malathion	20–60	$y = -3.339 + 1.7321x$	0.976	0.049

[a] Based on weighted linear regression; x = injected amount to GC, y = NPD detector responseas area

Table 2 Recoveries of compounds from the fortified cucumber samples at both F_1 and STB-F level, based on solvent calibration and both matrix matched calibrations

AP[a]	Solvent (isooctane) calibration				F_1 samples with matrix calibration/2.5 g seq		STB-F samples with matrix calibration/0.25 g seq	
	F_1 samples		STB-F samples					
	Chlorpyrifos	Malathion	Chlorpyrifos	Malathion	Chlorpyrifos, Q_R^b	Malathion, Q_a^b	Chlorpyrifos	Malathion
1	52.11	63.10	49.64	48.68	60.54	64.66	66.01	54.16
2	50.07	62.67	62.03	68.76	58.29	64.21	83.95	82.71
3	53.37	67.39	70.33	61.49	61.93	69.17	95.98	72.36
4	70.20	75.02	77.70	49.31	80.51	77.20	106.66	55.05
5	44.84	58.28	49.23	43.96	52.51	59.58	65.41	47.44
6	61.52	65.99	60.56	52.88	70.92	67.71	81.82	60.13
7	61.44	62.43	50.01	44.56	70.84	63.96	66.54	48.29
Av	56.22	64.98	59.93	52.80	65.08	66.64	80.91	60.02
S_d	0.086	0.059	0.112	0.092	0.095	0.056	0.162	0.13
RSD	15.29	8.14	18.63	17.38	14.58	8.35	19.99	21.74

[a] Analytical portion

[b] The definitions of Q_R and Q_a are given in Eq. 2

calibration and corresponded to matrix calibration/0.25 g seq respectively, for STB-F samples. The same situation was also valid for malathion. That is why quantitation of residue was performed matrix matched calibration which based on sample content of the injected extract volume. On the other evaluation, the recovery of fortified F_1 samples is lower than fortified STB-F samples for chlorpyrifos. The reason may be the higher sample mass of F_1 samples in the injected volume.

The assessment of stability studies were done based on various references (Anonymous 2005; and Personal communication with Dr A. Ambrus-Center for Plant Protection and Soil Conservation, Budaörsi, Hungary: Calculations for stability testing). The fortification mixture prepared for the surface treatment contained the residues of malathion and the reference compound which is indicated with *R*. The expected residue of the analyte (*A*) is calculated with the Eq. 2.

$$A = (R_{ref} a Q_a)/Q_R \quad (2)$$

where R_{ref} is the expected residue of reference compound. The *a* value is the ratio of the analyte and the reference compound in the treating solution, which is equal 1 in our cases since the both chlorpyrifos and malathion concentrations are the 0.1 μg/μL. Q_a and Q_R are the average analytical recoveries of analyte and reference compounds, respectively, which were determined from the fortified analytical portions (F_1, i.e., 0.02 mg/kg level) during method validation before the experiment.

The R_{ref} is calculated from its average recovery:

$$R_{ref} = R_{ref}'/Q_R \quad (3)$$

where R_{ref}' is the measured/survived residue of reference compound.

The stability of residues during sample processing was assessed by comparing the mean recovery of F_1 with the mean survival recovery of STB-F samples. Performing the STB-F analysis; the measured residue, expected residue which were calculated with the Eq. 3, and their reliability such as the standard deviation (S_d) and relative standard deviation (*RSD*%) between the 3 GC run were summarized in Table 3. The data indicated that S_d and *RSD* values between the injections are within the required limits (Anonymous 2006). As shown in Table 3, average differences (x_d) between the survived and expected residues, corresponding each measurement pairs, were calculated, and the average, and S_d of differences as well, by using Excel programme (Personal communication with Dr. A. Ambrus, Testing the stability of residues during sample processing). There was a consistent negative difference between measurement pairs.

To test significance of difference (t_{calc}), one-tailed *t* test was applied at 95% confidence level with the Eq. 4 (Miller and Ambrus 2005).

$$t_{cal} = \frac{\overline{x_d}}{s_d/\sqrt{n}} \quad (4)$$

where n is the total number of test.

The tabulated *t* values (t_{crit}) for checking the differences with the (n−1) = 20 degrees of freedom is 1.725. If the test statistics calculated with Eq. 4 is equal or lower than the critical value ($t_{calc} \leq t_{crit}$) the difference is not significant and the residue did not decompose. Since our $t_{calc} > t_{crit}$

Table 3 Calculated parameters for the assessment of stability of pesticides

Analytical portion	Measured residue, mg/kg		Expected residue[a], A mg/kg		Difference (malathion)	Test reliability			
	Ref. Comp, Chlorpyrifos, R_{ref}'	Test compound, malathion	Chlorpyrifos, R_{ref}	Malathion		S_d of Chlorpyrifos	S_d of Malathion	*RSD* (%) of Chlorpyrifos	*RSD* (%) of Malathion
STB-F1/1	0.126	0.098	0.193	0.198	−0.100	0.0068	0.0103	5.15	9.48
1	0.139	0.119	0.214	0.219	−0.101				
1	0.131	0.108	0.201	0.206	−0.098				
STB-F2/2	0.159	0.152	0.244	0.250	−0.097	0.0080	0.0130	4.77	7.88
2	0.172	0.178	0.265	0.271	−0.093				
2	0.173	0.166	0.266	0.272	−0.106				
STB-F3/3	0.172	0.128	0.265	0.271	−0.144	0.0170	0.0148	8.84	10.20
3	0.201	0.154	0.309	0.316	−0.162				
3	0.203	0.152	0.311	0.319	−0.167				
STB-F4/4	0.202	0.099	0.310	0.318	−0.218	0.0112	0.0098	5.27	8.88
4	0.224	0.118	0.345	0.353	−0.234				
4	0.214	0.112	0.329	0.337	−0.224				
STB-F5/5	0.123	0.087	0.189	0.194	−0.106	0.0067	0.0069	5.17	7.24
5	0.135	0.097	0.208	0.213	−0.117				
5	0.134	0.101	0.206	0.211	−0.110				
STB-F6/6	0.152	0.107	0.234	0.240	−0.133	0.0097	0.0111	5.95	9.25
6	0.171	0.127	0.263	0.269	−0.142				
6	0.167	0.127	0.257	0.263	−0.137				
STB-F7/7	0.132	0.090	0.203	0.208	−0.118	0.0036	0.0057	2.70	5.89
7	0.137	0.100	0.211	0.216	−0.115				
7	0.130	0.099	0.200	0.205	−0.105				
Average	0.162	0.120	0.249	0.255					
Average difference					−0.135				
SD of differences in malathion measurement					0.042				
Test for significance of difference: t_{calc}					14.541				
t_{crit}.0.05 –20					1.725				
RSD test comp. (%)					8.86				

[a] Expected residue of chlorpyrifos is the ratio of measured residues of STB-F samples to reference compound recovery which is determined F_1 fortification samples analysis. Expected residue of malathion is defined in Eq. 2, the definitions of R_{ref}' and R_{ref} in Eq. 3 as well

(14.541 > 1.725) it means the analyte decomposed during sample processing (Table 3). So, samples must be processed in the presence of dry ice, for the analysis of chlorpyrifos and malathion residues in cucumber.

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