DETERMINATION OF TEBUCONAZOLE AND PENCONAZOLE FUNGICIDES IN RAT AND HUMAN HAIR BY LIQUID CHROMATOGRAPHY/TANDEM MASS SPECTROMETRY

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ABSTRACT

RATIONALE: Tebuconazole (TEB) and penconazole (PEN) are widely applied fungicides and environmental contaminants; their toxicological properties include possible effects to the unborn child, therefore the evaluation of human exposure is relevant to risk assessment. Hair is a non-invasive specimen that incorporates pollutants allowing an extended exposure window to be surveyed. Aim of this work was to develop and validate an assay for the determination of TEB and PEN in human hair.

METHODS: Under optimised conditions, analytes were extracted soaking hair in acetonitrile, in the presence of deuterated analogues, under heating and agitation. Chemical separation was achieved using a C18 reversed-phase chromatographic column and detection and quantification were performed, after a positive electrospray ionization, by triple quadrupole mass spectrometry operating in selected reaction monitoring mode.

RESULTS: The assay validation showed a linear dynamic range up to 5 µg/L or 200 pg/mg hair, inter- and intra-run precisions <6%, and accuracies within 5% of spiked concentrations. Limits of quantification were 0.001 µg/L or 1 pg/mg hair for both TEB and PEN. Matrix effect experiments showed that the isotope dilution approach allowed for the control of bias sources. TEB and PEN were determined in hair of rats exposed to a low dose of TEB and in hair of agricultural workers exposed to TEB and/or PEN during the application season, indicating that both chemicals are incorporated into the hair upon exposure.

CONCLUSIONS: The results of this study indicate that the developed assay is useful to evaluate the exposure to TEB and PEN in humans.

INTRODUCTION

Tebuconazole and penconazole are conazole fungicides widely used in different crops, including grape, cereals and fruit trees. Due to their intentional spread into the environment, humans can be exposed during agricultural works and ingestion of contaminated food. The toxicological properties of these chemicals indicate possible effects for the unborn child,[1,2] therefore exposure evaluation is relevant for human risk assessment.

Biological monitoring of the exposure allows the determination of the body burden of a toxicant entering the human body from all routes and different sources. It is performed measuring a biological index, generally the chemical or a chemical's metabolite in easily accessible specimens. Blood and urine are the most commonly used specimens. In particular, urine, which can be easily obtained in large amount without invasive procedures, has been largely used in several investigations. In the frame of pesticides exposure, urinary chemicals are usually hydrophilic metabolites of the parent compound, often produced and eliminated within a few hours or days after exposure; therefore, urine biomonitoring is useful to assess short-term exposure.

Lately, hair has been increasingly considered as a relevant matrix for the biomonitoring of human exposure to environmental pollutants. Indeed, the possibility to reach extended windows of detection and to obtain information representative of the average level of xenobiotics entered into the body from a single specimen is particularly interesting in the context of chronic exposure biomonitoring. For this purpose, hair has been tested for several environmental pollutants such as metals, PCBs, and pesticides.[3-7] Among other advantages, there are the easy sample collection, that does not require medical staff and facilitates patient compliance. On the other hand, the little amount of hair typically available for the assay (tens to hundred milligrams), and the low concentration of xenobiotics in hair, require the use of highly sensitive analytical methods.

The assay for measuring pesticides in hair is often a multistep procedure that includes the decontamination of external chemicals, the extraction of incorporated chemicals, often using a solution of organic solvents, the chemical analysis of the extract. The analytical instrumentation is based on gas or liquid chromatography interfaced with mass spectrometry or tandem mass spectrometry; the recent improvement in instrumentation technologies allowed to set multi-class pesticide analysis with high selectivity and sensitivity.[6,8-9]

The aim of the present work was to develop and validate a method to detect and quantify

tebuconazole (TEB) and penconazole (PEN), both conazole fungicides, in human hair. In an explorative phase, we verified the presence of TEB in the keratin matrix of rats after parenteral administration. Secondly, we applied the developed assay to verify the presence of TEB and PEN in the hair of individuals exposed in agriculture.

EXPERIMENTAL

Chemicals

TEB and PEN (purity 98.7% for both, PESTANAL®, Analytical Standard, Sigma-Aldrich, Milano, Italy) were used for the preparation of standard solutions. Tebuconazole-d6 (TEB-d6, 99.7% atom D, Dr Ehrenstofer, LGC Standards, Milano, Italy) and penconazole-d7 (PEN-d7, 98% atom D, Sigma-Aldrich, Milano, Italy) were used for the preparation of the internal standard (IS) solution. For mobile phase, standard solution, assay optimization and sample preparation, methanol (MeOH), acetonitrile (CH_3CN), acetone, and aqueous acetic acid (99% purity, LC-MS/MS grade, Sigma-Aldrich, Milan, Italy) were used. Purified water was obtained using a Milli Q Plus ultra-pure water system (Millipore, Milford, MA).

Standard, calibration and quality control solutions

Standard solutions containing TEB and PEN were prepared in MeOH at concentrations of 100, 10 and 1 µg/L. An IS solution containing TEB-d6 and PEN-d7, each at 100 µg/L, was prepared in MeOH. Standard and IS solutions were stored at –20 °C in the dark. Under these conditions, the solutions were stable up to 6 months.

Calibration solutions (0.02, 0.04, 0.08, 0.2, 0.5, 1, and 5 µg/L for both TEB and PEN) and QC solutions (0.05 and 2 µg/L for both TEB and PEN, low- and high-QC, respectively) were prepared by adding suitable amounts of standard solutions to CH_3CN, in the presence of hair of non-exposed donors. An unspiked sample of hair in CH_3CN was used as blank. The concentrations, initially expressed in µg/L, were converted to pg/mg hair (50 mg hair samples were extracted with 2 ml CH_3CN). Therefore, the concentrations of the calibration solutions and QC solutions were 1.0, 1.6, 3.0, 8.0, 20, 40, 200 pg/mg hair and 2 and 80 pg/mg hair, respectively, for both TEB and PEN.

To optimize the hair extraction procedure, hair from healthy donors without occupational

exposure to fungicides was used. Before starting the analytical procedure, the IS solution was added to each calibration solution, QC solution and unknown sample to the final concentration of 2.0 µg/L (80 pg/mg hair) for both TEB-d6 and PEN-d7.

Equipment

For sample extraction and evaporation, a dry-block with nitrogen flow (Reacti-Vap Pierce, Milan, Italy) and an horizontal shaker with a rotatory vibration (Carlo Erba Reagents, Milan, Italy) were used. For LC-MS/MS analysis, 1.8 mL glass vials with screw caps (National Scientific, Superchrom, Milan, Italy) were used. Analysis was performed by high performance liquid chromatography (TurboFlow system, Thermo Scientific, Rodano, Italy) using a Betasil C18 column (150 mm length, 2.1 mm internal diameter, 5 µm particle size, Thermo Scientific, Rodano, Italy). The LC was interfaced with a triple quadrupole mass spectrometer (TSQ Quantum Access, Thermo Scientific, Rodano, Italy) equipped with a heated electrospray ionization source (H-ESI), operating with a metal needle.

LC-MS/MS analysis

The LC separation was performed with the column kept at room temperature, using an isocratic mixture of 0.5% aqueous acetic acid/MeOH (30:70) as eluent flowing at 0.25 mL/min. The mass spectrometer was operated using the H-ESI in the positive ion mode. The ionization source parameters were: spray voltage 4500 V, ion transfer tube temperature 212°C, vaporizer temperature 246°C, nitrogen as sheath gas and auxiliary gas operating at the pressure of 30 and 5 units (arbitrary scale), tube lens offset 104 V and skimmer offset -6 V. Collision-induced dissociation was performed using Ar as the collision gas at a pressure of 1.5 mTorr (0.2 Pa) in the collision cell. Quantification was based on selected reaction monitoring (SRM) following the transition: m/z 308 → 70 and m/z 308 → 125 as quantifier and qualifier ions for TEB; m/z 314 → 72 and m/z 314 → 127 as quantifier and qualifier ions for TEB-d6; m/z 284 → 70 and 284 → 159 as quantifier and qualifier ions for PEN; m/z 291 → 70 and 291 → 160 as quantifier and qualifier ions for PEN-d7 (see Figure 1).

Hair sample preparation

A sample of 3 cm of the lock of hair, measured starting from the root, with an approximate weight of 50-100 mg, depending on the density and length, was added with 2 mL H_2O and vortexed at room temperature in a glass vial to remove contaminants on the hair surface. The aqueous

solution was then removed using a glass pipette and analyzed to control the presence of TEB or PEN. Rinsed hair was dried at 60 °C for one hour, than cut into small pieces with metal scissors, introduced into a 2 ml cryogenic tube (Eppendorf, Safe-Lock tube, Milan, Italy). The tube was placed in the grinding jars and was cooled down placing in a bath with liquid nitrogen for about 15 min; the sample was milled in the presence of 3 steel balls using a ball mill (MM400, Retsch Italy, Torre Boldone, Italy) operating at frequency of 25 Hz for 2.5 min.

About 50 mg of hair powder was transferred into a 4 ml glass vial with a screw cap. Hair powder was added with 2 mL CH_3CN; the cap was sealed with Parafilm® and the sample was extracted at 45 °C with a horizontal shaker with a rotatory vibration, operating at 150 rpm, for 3 hours. An aliquot of the extract (0.5 mL) was added with 5 µL of IS solution and this sample was dried at room temperature under a gentle stream of N_2. 50 µL of CH_3CN were used to reconstitute sample and 20 µL of this solution were injected onto the LC injection port and analyzed with the procedure described above.

The concentration of TEB and PEN in the extract, initially measured in µg/L, was converted to pg/mg hair by taking into account the weight of the hair sample (mg).

Set up of the analytical sequence

In routine analysis, the calibration curve and QC were run with every set of unknown samples. A typical analytical sequence consisted of a calibration curve followed by unknown samples and one low- and one high-QC (0.05 and 2 µg/L, respectively) every ten unknown samples, followed by a second calibration curve.

Method Development

LC-MS/MS analysis

MS/MS working conditions, such as H-ESI parameters, SRM transitions and collision energies, were optimized by direct infusion of standard solutions in MeOH (5 mg/L) using a combination of manual and auto tuning. Signals were registered in the positive ionization mode. Full scan mass spectra were acquired keeping Q1 locked on the m/z corresponding to the protonated analyte molecules. Spray voltage, ion transfer tube temperature, vaporizing temperature, sheath gas and auxiliary gas pressure, tube lens offset and skimmer offset were optimized in order to obtain the most abundant MS/MS transition. To optimize the LC separation, the effect of different

combinations of 0.5% aqueous acetic acid/MeOH on peak separation, peak shape, retention times and signal to noise ratio, was determined.

Extraction of TEB and PEN from hair

Exploratory experiments were performed using hair samples (about 50 mg) from donors exposed to fungicides. These samples were extracted with different solvents (water, acetone, MeOH, and CH_3CN), extraction methods (sonication, heating); temperatures (room temperature, 45, 60 and 75 °C); times (1, 2, 3, 5 and 24 h). Additional tests to evaluate extraction completeness and reproducibility were done. Each experiment was performed in duplicate or quintuplicate, for reproducibility. Analysis of the extracts was performed using the LC-MS/MS procedure described above.

Assay validation

Calibration curve, limits of detection and quantification, precision, accuracy, and mid-term stability

Calibration curves were obtained in the presence of hair of non-exposed donora. A blank sample and seven non-zero calibration solutions covering the expected range of concentrations were used. Least squares linear regression analysis was applied to interpolate the data pairs obtained from each calibration solution, where *y* was the ratio between the chromatographic peak area of each analyte and the chromatographic peak area of the corresponding deuterated analyte and *x* was the concentration (µg/L) of each analyte. For method development, six replicates of each calibration level were analyzed.

The limit of detection (LOD) was evaluated as the concentration corresponding to three times the standard deviation of the signal in the blank sample.

The limit of quantification (LOQ) of the assay was calculated according to the expression:

$$LOQ = (5SE_q + q)/m$$

where SE_q is the standard error of the intercept *q* and *m* the slope of the linear regression.[10]

Intra- and inter-day precision and accuracy were determined by analyzing low- and high-QC solutions, three times on the same day and on five different days over a period of 6 months,

respectively. Precision was expressed as the coefficients of variation (RSD%). Accuracy was calculated as the percent ratio between the concentration calculated from the calibration curve and the theoretical (spiked) concentration (%Theoretical).

Mid-term stability was evaluated as the variability of the calibration curve slopes (n=6) over a period of six months, and estimated as $RSD\%_{slope}$.

Matrix effect and selectivity

A matrix effect is defined as the direct or indirect variations in analyte response due to the combined effect of all components of the different biological samples and the unintended presence of analytes of interest or other interfering substances in the sample.[11] The determination of matrix effects is an important aspect of biological analysis. In this study, the matrix effect was determined in different ways. Hair samples from five different donors belonging to the general population with different hair color were selected. Hair samples (50 mg each) were soaked in 2 mL of CH_3CN. TEB and PEN were added to each hair-CH_3CN sample to obtain calibration solutions and low- and high-QC solutions (each solution was prepared in duplicate).

% $Matrix_{proc}$ was determined by comparing the chromatographic signal of TEB and PEN in the hair extraction sample with that obtained in CH_3CN solution (without hair), after having submitted both samples to the extraction procedure. % $Matrix_{proc}$ was calculated by the formula:

$$\% \ Matrix_{proc} = ([TEB \text{ or } PEN_{hair}]/[TEB \text{ or } PEN_{CH3CN}]) * 100$$

Relative matrix effect (% $Matrix_{relative}$) was determined as the inter-matrix precision value (expressed as RSD%) of the QC solutions and of the slopes of the calibration curves. Morever, the inter-matrix slope range (% R_{slope}) was calculated by the formula:[12]

$$\% \ R_{slope} = [(Maximum_{slope} - Minimum_{slope})/Maximum_{slope}]*100$$

where $Maximum_{slope}$ and $Minimum_{slope}$ are the highest and the lowest slope value of the calibration curves obtained from the different subjects. All calculations were performed both with and without IS adjustment for comparison.

To investigate the selectivity of the assay, the interfering effect of co-present pesticides commonly used in the vineyards was investigated. With this aim a mixture of 20 pesticides (bitertanol, oxifluorfen, zoxamide, azoxystrobin, bupirimate, cyprodinil, fludioxinil, indoxacarb,

chlorpyrifos, cyazofamid, dimethomorph, iprovalicarb, metrafenone, quinoxyfen, cymoxanil, fenamidone, spiroxamin, metalaxyl-M, mancozeb, pyrimethanil) was added to hair samples, to obtain a solution containing each chemical at the final concentration of 2 µg/L or 80 pg/mg hair. Analysis of samples was performed using the LC-MS/MS procedure described above.

Analysis of real hair samples

Rats

Four CD1 male rats (Charles-River, Calco, Italy) was used for the experiment. Each rat had an homogenous body weight (BW) of 150 g. Before starting treatment a pre-treatment hair sample (T0) of the dorsal region (an area of about 10 cm^2 between the shoulder blades, where it was impossible self-licking), was obtained by shaving. Animals were kept in individual metabolic cages with a wire floor, at 12 dark/light cycle and were fed with food pellets (Italiana Mangimi) *ad libitum*. Animals received TEB dissolved in ethanol (5 mg/kg BW) by gavage once a day for two days. The fourth day, when the regrowth of hair was almost complete, but the shaved area was still identifiable, rats were sacrificed and post-treatment hair samples were collected from the area previously shaved (T1). The administered dose was chosen as a reasonable compromise between the absence of toxicity (TEB NOEL 10 mg/kg BW per day),[13] and the chance to observe a significant uptake in rat hair. The administration route and the experimental conditions were chosen to limit as much as possible external contamination of hair. Samples were analyzed as described in the procedure reported above.

Humans

Hair samples from 4 vineyard workers using TEB and/or PEN were analyzed. Agricultural workers mixed, loaded and applied TEB and/or PEN in the form of a diluted oil-in-water emulsion for foliar application on grapes either with a tractor-mounted air blast application. Samples were collected at the end of the application season, cutting a lock of hair of about 3 cm length, from the occipital region of the head. A lock of hair, cut as close as possible to the root using fine scissors, was attached to a paper sheet with masking tape with root-tip direction indicated and stored at room temperature in the dark until analysis.

RESULTS AND DISCUSSION

Method development

LC-MS/MS analysis

Optimum tuning parameters and chromatographic conditions are summarized in Method development. Signals were registered in the positive ionization mode; for each chemical, the transitions producing the most abundant ion, involving the formation of the triazole residue from the molecular ion, was chosen for quantification: m/z 308 → 70 (TEB); m/z 314 → 72 (TEB-d6); m/z 284 → 70 (PEN); m/z 291 → 70 (PEN-d7). The transitions producing the second most abundant ion,s involving the formation of 4-chlorobenzil or 2,4-chlorobenzil residues from the molecular ions, were chosen as qualifiers: m/z 308 → 125 (TEB); m/z 314 → 125 (TEB-d6); m/z 284 → 160 (PEN); m/z 291 → 160 (PEN-d7) (see Figure 1). The retention times of TEB and PEN (mean ± SD) were 13.25 ± 0.23 min and 12.51 ± 0.33 min (n = 50 injections), respectively.

Extraction of TEB and PEN from hair

After a decontamination step, performed with water at room temperature, hair was milled to a fine and homogeneous powder, and extraction experiments were performed using about 50 mg of hair powder. Compared to other system to treat hair, hair powder has larger surface available for extraction and avoids difficulties of handling small bits of hair, typically electrically charged and repelling each other.

To identify the best conditions to perform the extraction of conazoles, hair samples of exposed donors were used. This approach is alternative to the use of hair spiked with known amount of pesticides, the use of which may be misleading, given the different nature of the chemical binding in real hair samples, enriched by the absorption of exogenous substances from the blood stream, sweat and/or sebaceous excretions during hair growth, and the deposition on the hair surface that occurs using spiked hair samples.[14,15] Performing extraction experiments using hair of exposed subjects is therefore mandatory to get realistic data. On the other hand, using such samples, the true concentration of the analytes incorporated in hair is unknown. Therefore, the recovery can only be estimated as a relative recovery, comparing different extraction media and conditions, and taking the most effective condition as the reference.

In our study, the best extraction media was found to be CH_3CN (highest recovery or 100%). Water yielded a relative extraction efficiency of ~ 30%. MeOH and acetone showed good extraction efficiencies, 88 and 98% respectively, but their high volatility, especially for acetone, caused a fast

evaporation during heated extraction. Overall, CH_3CN was selected as the best extraction solvent; this is in line with a previously reported method.[8]

Sample stirring and heating improved extraction efficiency. The extraction temperature of 45 °C was finally chosen, because at higher temperature a pronounced solvent evaporation was found. The extraction time was set at 3 h, as a compromise between the maximum extraction efficiency, obtained at 5 h (100%), and a convenient preparation time. The extraction efficiency at 3h was 94 ± 2%.

To ensure the complete extraction, a total of 6 consecutive extraction steps on the same hair sample were performed. The large majority of the analyte was extracted in the first step; amounts of about 7 and 3 % were found in the second and in the third step. Overall, considering the effort to perform a second extraction and the small gain of analyte associated with it, in the final protocol a single extraction step was set.

Under the defined protocol, the repeatability of the extraction was good, with a RSD% < 3%.

Assay validation

Calibration curve, limits of detection and quantification, precision, accuracy, and mid-term stability

A summary of the validation parameters is reported in Table 1. Good linearity was found for both TEB and PEN over the investigated range, with coefficients of determination (R^2) higher than 0.994. For both analytes LOD and LOQ were 0.8 and 1 pg/mg hair, respectively. Precision and accuracy at LOQ were 3.5% and 7.4 %, and 98% and 104% for TEB and PEN, respectively. Inter- and intra-run precision and accuracy of the assay met the US FDA requirements for the validation of bioanalytical methods (precision, estimated as RSD% < 10%; accuracy between 97 to 103% of the spiked concentrations). At each calibration point, RDS% of ranged from 0.8 to 3.8 and 0.5 to 7.4 for TEB and PEN, respectively; the mean accuracy was 100 and 99 for TEB and PEN, respectively. For both TEB and PEN mid-term stability of the calibration curve was good, with $RSD\%_{slope}$ below 4%, that is within the range of intra-day precision. These results are comparable or better than those previously reported for TEB in a multi-class pesticides method using direct injection solid phase microextraction or liquid injection followed by and gas chromatography-tandem mass spectrometry.[8]

Matrix effect and selectivity

The results obtained in the evaluation of the matrix effect are reported in Table 2. For PEN, the $\%Matrix_{proc}$ showed a significant matrix effect, with a value of 169% for low-QC solutions. The matrix effect was completely corrected using the deuterated analog. On the contrary, TEB did not show the same effect. The variability of low- and high-QC responses in different matrices ($\%Matrix_{relative}$) gave precisions (%RSD) ranging from 23.0 to 33.2% without IS correction; with IS the %RSD values ranged from 2.5 to 3.7%. These results confirmed the absence of a matrix effect when the IS was used.[12] Finally, the $\%R_{slope}$, representing the maximum difference in the slopes of the linear calibration curves obtained using matrices from different individuals, ranged from 28.9 to 31.3% without IS and was reduced below 3.2% after adjustment with IS. Overall, some matrix effect was observed for the determination of TEB and PEN, however, corrections using deuterated analogs as IS balanced this effect and improved the analytical performance.

The selectivity experiment showed that there was no interference by any other pesticides applied on vineyard during the application season, in the chromatograms of TEB and PEN.

Analysis of hair samples

Rats

In Table 3 the results of the experiment performed with rats are reported. No signal of TEB or PEN was found in the solution used for the decontamination step. Similarly, no TEB was found in T0 samples, while it was found in T1 samples. A mean TEB content of 260.8 (±30.4) pg/mg in post-treatment hair was measured. On the basis of hair weight and the corresponding shaved area, a hair density of 10 mg/cm^2 was estimated. Calculating a total surface area of about 200 cm^2, according to Diack's formula:[16]

$$(7.47 \times \text{Body weight})^{0.66}$$

a total of about 2000 mg hair/rat was estimated. Considering the average TEB level in the samples, it was calculated that, in the hair from the entire rat, there would be about 521556 (± 6081) pg TEB, which corresponds to an incorporation rate of about 0.10% (± 0.012%). This finding is compatible with those of previous studies conducted administering pesticides to rats.[17-19]

Taking into account that rats were kept in individual cages, that the shaved area was on the

dorsal region, where rats couldn't self-lick, and considering that TEB is excreted in urine after an extensive biotransformation, it can reasonably be excluded any contamination of hair from both saliva and urine.[20]

Human

In Table 3 the results of the analysis of hair of four vineyard workers are summarized. No signal of TEB or PEN was found in the solution used for the decontamination step. This was expected, as the post exposure sample was collected at the end of the application season, that was weeks or even months after the last application of fungicides in the vineyards. In this condition, any external contamination was removed by personal hygienic procedures. In all human samples, TEB and PEN were detectable. Mean concentrations of 143.1 pg/mg hair of TEB and 70.1 pg/mg hair of PEN were found in workers who applied fungicides. Mean concentrations of 19.4 pg/mg hair of TEB and 18.8 pg/mg hair of PEN were found in workers who did not apply fungicides. This reveals that a contamination of both conazoles is present in the agricultural environment. The levels of TEB in non-applicators is in line with 37 pg/mg hair, previously reported in 2 out of 14 volunteers belonging to a laboratory stuff.[8]

CONCLUSION

A LC-MS/MS method for the determination of the fungicides TEB and PEN in hair was developed and validated. Good linearity, precision and accuracy were obtained. Moreover, the high specificity and sensitivity of the developed method enabled the quantification of TEB and PEN in real samples. The use of specific, labelled internal standards plays an essential role in controlling sources of bias. Our results indicate that TEB and PEN are incorporated into the hair of both rats and humans. This result shows that the proposed assay is suitable to measure PEN and TEB in human hair for the evaluation of cumulative exposure to conazole pesticides.

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Table 1. Calibration curve data, limit of detection (LOD), limit of quantification (LOQ), precision and accuracy of the assay.

Analyte	Investigated ranges	Calibration curve					QC precision and accuracy				
		RSD%$_{slope}$ [a]	LOD	LOQ	RDS% at LOQ level	%Theoretical at LOQ level	Spiked concentrations	Day 1-5 n=3		Overall n=15	
								RSD% [b] (Min-Max)	%Theoretical [c] (Min-Max)	RSD% [d]	%Theoretical [c]
TEB	**1-200 pg/mg hair**	3.2	0.8 pg/mg hair	1.0 pg/mg hair	3.5	98	Low	2.8 (0.9-4.7)	101 (99-102)	3.0	101
							High	2.1 (0.5-3.5)	101 (98-103)	2.6	101
PEN		1.9	0.8 pg/mg hair	1.0 pg/mg hair	7.4	104	Low	3.7 (0.7-5.0)	100 (97-103)	4.1	100
							High	3.3 (2.3-4.3)	100 (98-101)	3.1	100

[a] Mid-term stability of calibration curves
[b] Within-run precision
[c] Accuracy
[d] Between-run precision

Table 2. Matrix effect of the process (%Matrix$_{proc}$), relative matrix effect (%Matrix$_{relative}$), and inter-matrix slope range (%R$_{slope}$) of the assay.

Analyte	QC					Calibration curves			
	QC Level	%Matrix$_{proc}$ without IS (SD)	%Matrix$_{proc}$ with IS (SD)	%Matrix$_{relative}$ without IS	%Matrix$_{relative}$ with IS	%Matrix$_{relative}$ without IS	%Matrix$_{relative}$ with IS	%R$_{slope}$ without IS	%R$_{slope}$ with IS
TEB	**Low**	93 (33.2)	108 (2.7)	33.2	2.5	11.7	1.4	28.9	3.1
	High	88 (20.8)	103 (3.2)	23.6	3.1				
PEN	**Low**	169 (51.9)	96 (3.5)	30.8	3.7	10.8	0.9	31.1	2.3
	High	102 (26.6)	99 (3.5)	23.0	3.6				

Table 3. Percentage of quantifiable samples and levels of TEB and PEN in hair samples of rats and agricultural workers.

		Rats		Agricultural workers	
		T0	**T1**	**TEB applicators**	**PEN applicators**
TEB (pg/mg hair)	% samples ≥ LOQ	0	100	100	100
	Mean (±SD)	< 1	260.8 (30.4)	141.3 (147.0)	18.8 (10.7)
PEN (pg/mg hair)	% samples ≥ LOQ	Np	Np	100	100
	Mean (±SD)	Np	Np	19.4 (18.0)	70.1 (19.1)

Np= experiment not performed