Assessment of penconazole exposure in winegrowers using urinary biomarkers

Rosa Mercadante[a], Elisa Polledri[b], Federico Maria Rubino[c], Stefan Mandic-Rajcevic[c], Andrea Vaiani[c], Claudio Colosio[c], Angelo Moretto[d], Silvia Fustinoni[§a,b]

[a] *Department of Clinical Sciences and Community Health, University of Milano, Via S. Barnaba 8, 20122 Milan, Italy*

[b] *Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Via S. Barnaba 8, 20122 Milan, Italy*

[c] *Department of Health Sciences of the University of Milan, Laboratory for Analytical Toxicology and Metabolomics (LaTMA) and International Centre for Rural Health of the University Hospital San Paolo, Via di Rudini 8, Milan, Italy*

[d] *Department of Biomedical and Clinical Sciences, University of Milano, and International Centre for Pesticides and Health Risks Protection (ICPS), "Luigi Sacco" Hospital, Via G.B. Grassi 74, 20157 Milano, Italy*

Corresponding Author:

[§]Authors' correspondence address:

Via S. Barnaba, 8 – 20122 Milan, Italy

phone: +39 02 503 20158

fax: + 39 02 503 20111

e-mail: silvia.fustinoni@unimi.it

Abstract

Penconazole (PEN) is a fungicide used in agriculture. The aim of this work was to evaluate the exposure to PEN in vineyard workers focusing on urinary biomarkers.

Twenty-two agricultural workers were involved in the study; they were investigated during PEN applications and re-entry work, performed for 1-4 consecutive working days, for a total of 42 mixing and applications and 12 re-entries. Potential and actual dermal exposure, including hand exposure, were measured using pads and hand washes. Urine samples were collected starting before the first application, continuing during the work shift, and ending 48 hours after the last shift. The determination of PEN in dermal samples and PEN metabolites in urine was performed by liquid chromatography tandem mass spectrometry.

Dermal potential body exposure and actual total exposure showed median levels ranging from 18 to 3356 and from 21 to 111 µg, respectively. Urinary monohydroxyl-derivative PEN-OH was the most abundant metabolite; its excretion rate peaked within 24 h after the work shift. In this period, median concentrations of PEN-OH and the carboxyl-derivative PEN-COOH ranged from 15.6 to 27.6 µg/L and from 2.5 to 10.2 µg/L, respectively. The concentration of PEN-OH during the work shift, in the 24 h after and in the 25-48 h after the work shift were correlated with actual body and total dermal exposure (Pearson's r from 0.279 to 0.562).

Our results suggest that PEN-OH in the 24 h post-exposure urine is a promising candidate for biomonitoring PEN exposure in agricultural workers.

Keywords: urinary penconazole metabolites; biomonitoring; vineyard workers; dermal exposure; pesticides.

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1. Introduction

Vineyards are extensively present in Italy; of the total utilized agricultural area of about 12.9 million of hectares, which represents 43% of the Italian territory, grapes account for about 5%, with 664.296 hectares (ISTAT, 2010).

Grapevine is subject to the attack of molds, and fungicide pesticides are widely used to control these pests. According to European Union Statistical Office in 2015, about 38.861 tons of fungicides and bactericides were sold in Italy (EUROSTAT, 2015). Among fungicides, conazoles are triazole fungicides that interfere with the biosynthesis of sterols in fungi cell membranes. They are largely used on grapes, and within the class, penconazole (PEN, (RS)-1-[2-(2,4-dichlorophenyl)pentyl]-1H-1,2,4-triazole, CAS number 66246-88-6) is one of the most widely used.

The WHO/FAO Joint Meeting on Pesticide Residues set an acceptable daily intake (ADI) of 0.03 mg/kg body weight/day for PEN, this was (WHO/FAO, 1992) confirmed by the European Food Safety Authority Scientific Report (EFSA, 2008), and recently further reiterated by a new version of the WHO/FAO document (WHO/FAO, 2016). From the toxicological point of view, PEN has low acute toxicity and causes liver toxicity in experimental animals (mice, rats and dogs); PEN is not carcinogenic, nor genotoxic (WHO/FAO, 2016). Reduced mating index and longer gestation duration was observed in rats. In addition, PEN was found to be teratogenic (microphthalmia and hydroencephalus) in rabbits, but not in rats (WHO/FAO, 2016).

According to the harmonized classification and labelling approved by the European Union, PEN is very toxic to aquatic life with long lasting effects (hazard statements H400 and H410), is harmful if swallowed (H302) and is suspected of damaging fertility or the unborn child (H361) (Regulation (EC) No 1272/2008).

Given the use of PEN as a pesticide, and therefore its intentional spread into the environment, agricultural workers, agricultural families and rural residents living in the area surrounding treated

crops, and the general population consuming contaminated crop products and drinking water may be exposed. According to EU, the maximum residue level for PEN on table and wine grapes is 0,2 mg/kg (Commission Regulation (EC) No 149/2008).

Biological monitoring of exposure measures the body burden or the pool of a substance in the body, mostly by quantifying, as biomarker, the substance itself or a suitable bio-transformation product in an easily accessible biological compartment (Angerer et al., 2007; Berode et al., 2011). Recently, the metabolism of PEN in humans was investigated (Mercadante et al., 2016; Sams et al., 2016), showing that the major urinary metabolites are the monohydroxyl-derivative PEN-OH, mostly excreted as glucuronide conjugated, and the carboxyl-derivative PEN-COOH, although with a minor contribution (see Figure 1). Based on this result, we suggested the measurement of PEN-OH concentration in urine, after the hydrolysis of the glucuronide conjugate, for the biological monitoring of PEN exposure (Mercadante et al., 2016).

The aim of this work was to study the behavior of PEN-OH and PEN-COOH as biomarkers of PEN exposure. Twenty-two agricultural workers applying PEN in vineyards were recruited. External exposure was assessed collecting information on the use of PEN and investigating dermal exposure. In fact, dermal exposure was recognized as the main exposure route both during pesticide mixing, loading and application, and during re-entry activities (Aprea et al., 2005; Baldi et al., 2014; Baldi et al., 2006; Flack et al., 2008). PEN metabolites were measured in urine sampled before, during and after the application/re-entry in the treated vineyards.

2. Material and methods

2.1 Study subjects

The field study was performed from May to July 2012. The involved subjects were 22 vineyard workers who sprayed PEN in vineyards and performed post application activity (re-entry). They were recruited from different areas of Lombardy, Italy: ten workers were farm owners of the provinces of Bergamo and Brescia, and twelve workers were agricultural cooperative employees from the province of Sondrio. Agricultural workers mixed, loaded and applied PEN in the form of a diluted oil-in-water emulsion (about 10% w/w) for foliar application on grapes; application was performed with a sideways-spraying tractor-mounted air blast, or spraying upwards with hand-held application equipment. Re-entry workers were involved in monitoring access of grapes to air and sunlight, i.e. cutting buds in excess, removing young plant shoots or excess bunches of grapes, raising or lowering the wires. A total of 42 mixing and applications (M&A) and 12 re-entries (R) were performed. Study subjects worked in vineyards from 1 to up to 4 consecutive daily work shifts.

The research team administered the questionnaire, supervised fieldwork and collected data on personal characteristics, such as age, height, body weight, and occupational information useful to explore determinants of exposure. The latter included: the name and brand of the formulation; the concentration of PEN in the formulation; the size of the treated area; the quantity of formulation applied per area; the work shift duration; the application equipment and techniques; any maintenance of the machinery; the type of clothing and personal protective equipment worn during the work.

The study was performed in the context of the risk evaluation, according to Italian law for health and safety at the workplace (Decreto Legislativo 9 aprile 2008). A field staff that included technical and sanitary professionals performed the study. Each study subject read, understood and

signed the informed consent form, also with reference to yielding urine samples for biological monitoring.

2.2 Dermal sampling

Dermal exposure was assessed by the use of pads and the coordinated collection of the hand wash liquid. Body exposure was assessed using square 0.01 m^2 lab-made pads prepared with filter paper (Whatman no.1 filter paper, Prodotti Gianni, Milan) as previously described (Mandic-Rajcevic et al., 2018; OECD, 1997; Rubino et al., 2012). Pads were applied on six representative body districts, namely: right upper limb (arm + right forearm), left upper limb (arm + left forearm), front trunk (thorax + abdomen), rear trunk (back + loin), right lower limb (thigh + right shin), and left lower limb (thigh + left shin). Twelve pads were applied for each subject for the whole work shift, six of which above clothes and six under clothes; for each body district two different positions were selected to avoid superimposition. At the end of the work shift, each dermal pad was detached from clothes/skin, coded and kept in the dark at room temperature. In total, more than seven hundreds dermal samples were collected. To assess hand exposure, a modification of the "Hand rinse method" reported in the OECD guidelines (OECD, 1997) was used; in particular 100 ml of a solution of isopropyl alcohol in water (25% v/v) were used; this solution was chosen for its optimum solvent property and the low toxicity. In particular, a member of the research staff slowly poured the washing solution over the cupped hands of the worker for about 30-60 s while the worker rubbed his hands together. Hand wash liquid was collected in a basin underneath and then poured in a dark coded glass bottle, and stored at room temperature. The recovery of PEN during this operation was over 90%. The wash was performed at the end of the work with pesticides and/or at any other work break during the shift, according to subject's need. About 130 hand wash samples were collected. All samples were delivered to the laboratory within one week of collection; once in the laboratory, samples were kept at – 20 °C until analysis, that was performed within 6 months.

Pads on clothes were used to estimate the potential body exposure, defined as the amount of pesticide deposited on work clothes and/or coveralls. Pads under the clothes were used to estimate actual body exposure, defined as the amount of pesticide deposited on worker's skin, and that is the amount of pesticide able to reach the uncovered skin and available for absorption. Finally, actual total exposure was calculated as the sum of actual body exposure *plus* hand exposure.

2.3 Urine collection for biological monitoring

Urine samples were self-collected by workers during the study period. To this aim, workers were provided with instructions and a sampling kit consisting of sampling bottles and a collection sheet to record sampling time. Each subject was asked to collect pooled urine sample for the following periods:

- during the 24 h before the first work shift with the use of PEN (Pre-WS) (Day 0);
- during the work shift/s with PEN exposure due to M&A and/or R (Day 1 to 4);
- after M&A and R work shift/s, for 24 h or for a shorter period, for those who performed multiple consecutive work shifts (Post 24h/Post 24h-R) (Day 1 to 4);
- for additional 24 h after the Post 24h sample, until 48h after the last exposure (Post 25-48h) (Last day).

Samples were kept at room temperature, 20 - 25 °C, and retrieved, by study staff, at the end of each day. Urine volume was measured (± 5 mL) and an aliquot was taken and cooled to 4 °C for the analysis. Aliquots were delivered to the laboratory within 72 h after the end of collection. In the laboratory, aliquots were stored at -20 °C until analysis, which occurred within 6 months. The number of urine samples collected by each subject varied, based on the number of consecutive working days; all together 148 urine samples were obtained.

2.4 Measurement of PEN and PEN metabolites

For the preparation of standard solutions, PEN (purity 98.7%, Sigma-Aldrich, Milan, Italy), PEN-OH, and PEN-COOH (purity 98% for both, kindly donated by Syngenta, UK) were used. For

the preparation of the internal standard solution (IS), penconazole-d7 (98 atom % D, PEN-d7, Sigma-Aldrich, Milan, Italy) was used.

The measurement of PEN on dermal samples (pads) was performed after the desorption overnight at room temperature with 8 mL of an aqueous solution of 25% isopropyl alcohol in the presence of penconazole-d_7 (98 atom % D, PEN-d_7, Sigma-Aldrich, Milan, Italy) as internal standard. An aliquot of each solution was analyzed by high performance liquid chromatography (Surveyor, Thermo Scientific, Rodano, Italy) equipped with a Betasil C18 column (150 mm length, 2.1 mm internal diameter and 5 µm particle size; Thermo Scientific, Rodano, Italy) kept at room temperature, using an isocratic mixture of aqueous formic acid (0.5%) and methanol (30:70) at 250 µl/min as eluent. The liquid chromatography was interfaced with a LC-MS/MS (TSQ Quantum Access, Thermo Scientific, Rodano, Italy) equipped with a heated-electro spray ionization source. The extraction efficiency was about 98%. The intra- and inter-day precision of the method, assessed as a percent coefficient of variation, was less than 10%, the accuracy was 95-103%, and the limits of quantification (LOQ) was 1 µg/L. Field blanks, included to check the possible contamination of sampling material during the field study, showed no contamination.

The measurement of PEN metabolites in urine was performed as previously described (Mercadante et al., 2016). Briefly, urine samples were thawed at room temperature, added with a penconazole-d_7 solution, as internal standard, and β-glucuronidase hydrolysis solution. Hydrolysis was performed at 40 °C overnight. Analyses were performed as previously described (Mercadante et al., 2016), using a liquid chromatography-triple quadruple mass spectrometry (4500 System, SCIEX, Milano, Italy) equipped with a turbo ion spray (TIS) source interfaced with a binary pump (Shimadzu, Milano, Italy). The chromatographic separation was performed with a Hypersil Gold PFP column (50 mm length, 2.1 mm internal diameter, and 3 µm particle size; Thermo Scientific, Rodano, Italy) and with 0.1% aqueous formic acid (eluent A) and methanol (eluent B), at a flow rate of 500 µl/min. The analytes were detected in the positive ion mode and quantified based on

single reaction monitoring. The intra- and inter-day precision of the method, assessed as a percent coefficient of variation, was less than 10%, accuracy was between 100 and 108%, and the limits of quantification (LOQ) was 2 µg/L for PEN and 1 µg/L for both PEN-OH and PEN-COOH. For quality controls (QC), as no certified material was purchasable, in-house quality control solutions were prepared and analyzed together with samples in any analytical sequence. A typical analytical sequence consisted of analysis of zing calibration curve samples, followed by ten prepared unknown samples analyzed along with one duplicate sample, one low-QC, and one high-QC sample, followed by a second set of calibration curve samples (Mercadante et al., 2016). The throughput was about 50 samples/day.

Urinary creatinine was determined using Jaffe's colorimetric method (Kroll et al., 1986).

2.5 Data processing and statistical analysis

Calculations to estimate dermal exposure (µg) were performed as described elsewhere (Mandic-Rajcevic et al., 2018; Mandic-Rajcevic et al., 2015).

Briefly, the percentages of body surface represented by each pad were calculated using the Mosteller formula (Mosteller, 1987), considering the proportions of body areas of a normal healthy male. The potential body exposure was calculated as the sum of regional exposures which were measured by external pads (on the clothes), taking into account the surface of the pad and the body region represented by each pad. The actual body exposure was calculated as the sum of regional exposures calculated from the amount of PEN measured in the skin pads multiplied by the surface of each region represented by the pads. The actual total exposure was calculated summing actual body exposure with the amount of PEN on hand.

To describe the kinetics of PEN-OH, data were expressed as excretion rate (µg/h), taking into consideration urine volume and the duration of urine collection.

In order to ascertain whether the workers collected all urine voids, since no information on incomplete urine collection was reported, we calculated the expected daily amount of urinary

creatinine $Creat_{exp}$, according to the formula (Knuiman et al., 1986):

$Creat_{exp}$ (g/day) = body weight (kg) X 24 (g/(day x kg))

From the ratio between measured and expected daily amount of urinary creatinine, the percentage of incomplete urine collection was estimated (results reported in Table 1). This percentage is however affected by a large degree of variability and only when it falls outside the range 60 – 140%, urine sample should be considered unreliable (Joossens JV, 1984).

Finally the level of PEN-OH and PEN-COOH were also expressed in µg/g creatinine.

Statistical analysis was performed using SPSS 25.0 for Windows (IBM SPSS Statistics). Values below the LOQ were assigned a value equal to 1/2 LOQ. For the descriptive analysis median, minimum and maximum values of the distribution were calculated; moreover, for PEN-OH and PEN-COOH the percentage of quantified samples was added.

The correlation between dermal exposure and the urinary concentration of PEN-OH in the different periods was evaluated using Pearson's correlation performed on log-transformed variables. Due to the limited number of available observations, they were grouped based on the time frame. M&A1, M&A2, M&A3+R1, R2 samples were pooled and considered all part of the M&A+R group; Post 24h-1, Post 24h-2, Post 24h-3, Post 24h-R1, and Post 24h-R2 samples were pooled and considered in the Post 24h group. Pooled data were considered as independent observations.

3. Results

3.1 Study subjects

In Table 1, a summary of personal characteristics of study subjects and some information on the work shifts with exposure to PEN is reported. All subjects, but one, were males, with median age, height and weight of 48 years, 175 cm and 78 kg, respectively. During urine collection workers excreted a median creatinine amount of 1.3 g/day (from 0.9 to 2.4 g/day), and this was estimated to be 71% of the expected amount; 5 out of 22 subjects had median creatinine level below 60%, and their urine samples should be considered incomplete. In particular 4 out of 5 subjects had median values between 50 and 60 % (almost acceptable) and just one subject had very low creatinine level, that was 38% of the theoretical value.

Eight workers applied PEN during one work shift, two workers applied PEN for two consecutive work shifts, and six workers applied PEN for three consecutive work shifts. Moreover, six subjects applied PEN for two consecutive work shifts and performed re-entry activities for other two consecutive work shifts. In general, applicators performed different tasks: prepared the fungicide mixture, loaded the tank of the sprayer, either tractor-mounted or hand-held, sprayed the pesticide, and cleaned the equipment. Re-entry growers were mostly involved in monitoring access of grapes to air and sunlight, i.e. cutting buds in excess, removing young plant shoots or excess bunches of grapes, raising or lowering the wires, cutting excess branches (Baldi et al., 2014). The quantity of PEN sprayed ranged from 26 to 1500 g, the treated area ranged from 0.4 to 11 ha, and the work shift duration ranged from 1 to 12 h.

3.2 Dermal exposure

The results of dermal exposure to PEN are reported in Table 2. The potential body exposure during the different work shifts ranged 4.2 to 9197.3 µg during M&A, and from 6.2 to 56.0 µg during R. A tiny amount of PEN passed through the work clothes/coverall, with actual body exposure ranging from 0.9 to 526.7 µg during M&A, and from 0.8 to 4.6 µg during R. Median

exposure on hands ranged from 12.1 to 109.3 µg, with maximum levels up to 6482.4 µg during M&A3. Body and hand exposure contributed differently to the actual total exposure, accordingly to work tasks. During M&A, potential body exposure was quite high, but the corresponding actual body exposure was from 1/100 to 1/1000 lower, showing that work clothes/coveralls afford a good protection. For workers performing M&A, hand exposure and actual body exposure similarly contributed to actual total exposure. During re-entry activities, potential body exposure was low, and consequently actual body exposure was very low; conversely, hand exposure was about two orders of magnitude higher, so that hand exposure resulted to be the major contributor of actual total dermal exposure. Total exposures in those performing re-entry activities was about 2-fold higher than in those performing M&A.

3.3 Biological monitoring and excretion rate

The duration of urine collection ranged from 4.5 to 31.5 h, with a minimum value during work shifts and a maximum on Day 0; urine volume ranged from 117 to 2842 ml. Although a general coherence between sample volume and sampling duration was found, a more detailed analysis performed using the daily amount of urinary creatinine (Table 1) lead us to conclude that the majority of study subjects properly collected urine samples.

In Table 3, ~~were reported~~ the concentration of PEN metabolites in urine (expressed as µg/L and µg/g crea), and the excretion rate of PEN-OH referring to samples obtained during both the work shift and the post working period, were reported for each investigated day. The median concentration of PEN-OH ranged from 8.0 to 27.6 µg/L, with a maximum concentration of 237 µg/L in Post 25-48h. Considering creatinine excretions, the median concentration of PEN-OH ranged from 8.2 to 20.2 µg/g crea, with a maximum concentration of 301 µg/g crea in Post 25-48h. PEN-OH was quantified in all samples, including during Day 0, although workers declared that they had not used PEN in the previous part of the application season.

The median concentration of PEN-COOH ranged from <1 to 10.2 µg/L, with a maximum

concentration of 54.1 µg/L in Post 24h-3. PEN-COOH was quantified in 43 to 100% of samples, with a minimum percentage during Day 0. Considering creatinine excretions, the median concentration of PEN-COOH ranged from 0.9 to 12.6 µg/g creat, with a maximum concentration of 64.8 µg/g creatinine in Post 25-48h.

Median ratio between PEN-OH and PEN-COOH was about 5 (3.8 - 8.8), highlighting that in humans PEN is mostly metabolized and excreted as PEN-OH, both as glucuronide conjugated and in the free form (see Figure 1) (Mercadante et al., 2016; Sams et al., 2016).

Median PEN-OH excretion rate ranged from 0.37 to 1.19 µg/h, with a maximum value of 7.36 µg/h in Post 25-48h. In the box-plot graph of Figure 2, the excretion rates are reported. An increase in the excretion rate was observed comparing PEN-OH rate in samples collected during the work shift (M&A/R) and in the 24 h after the work shift (Post 24h). Considering consecutive days, a trend toward increasing values was observed, passing from Day 0 to Day 3, suggesting an accumulation of PEN-OH following consecutive exposures. Starting from Day 4, a decrease in the excretion rate was observed, with a minimum value of 0.48 µg/h in the last day.

Comparing PEN-OH concentration during M&A vs. R, no large difference was found. This was unexpected as actual total exposure was almost double in those who performed re-entry than in those who performed M&A. This suggests that hand exposure was less efficient than body exposure in determining internal dose of PEN and/or that additional exposure via inhalation could have contributed to the internal dose for M&A.

3.4 Correlation between dermal exposure and urinary PEN-OH

In Table 4, Pearson's correlation coefficients between dermal exposure and the concentration of PEN-OH in the different time frames are reported. Considering PEN-OH (µg/L), significant correlations were found between actual body exposure, for all time frames, with Pearson's r ranging from 0.386 to 0.562 and p values ≤ 0.01. Significant correlations were also found between actual total exposure and PEN-OH during M&A+R and Post 24h samples, with a

Pearson's r of 0.279 and 0.310 respectively, and p values ≤ 0.05. Similar or better correlations were obtained when PEN-OH was adjusted for creatinine,

The scatter plot, linear regression line and confidence interval between actual body exposure and PEN-OH in the Post 24h, are reported in Figure 3. On the contrary, no significant correlation was found between PEN-OH and potential body exposure or hand exposure.

4. Discussion

Following a previous study in which PEN-OH, as glucuronide conjugate and in the free form, was identified as the major metabolite of PEN in human urine, this work shows that total PEN-OH excreted in urine is a useful biomarker of exposure to PEN. Moreover, it identifies the 24 h post exposure period as the best time for sample collection.

This study is very notable for many aspects. First, the study protocol entails simultaneous collection of dermal and urine samples, and results allow to correlate dermal exposure and urinary PEN-OH. Second, consecutive urine samples were collected starting before the first application of the season and continuing until 48 h after the end of the last work shift with exposure to PEN. This allowed investigating the formation of the main metabolites of PEN, and their respective kinetics of urinary elimination. Third, the study protocol followed workers across several days, where activities included consecutive spraying days and re-entry, and resting periods. This allowed comparing exposures associated to different tasks in agriculture.

As shown in Table 1, 22 study subjects experienced different exposure patterns, such as one to three consecutive application shifts, and the combination of applications and re-entries. This, together with the different application procedures, the variable amount of PEN applied, and the different working times, yield a wide spectrum of exposures, as reflected by the large variability of dermal potential body exposure that spanned over three orders of magnitude, from few μg up to almost 10 mg. As for actual body exposure, a reduction of PEN and its availability for absorption is observed, with values ranging from below 1 μg to 500 μg, therefore spanning over two orders of magnitude. The low amount of PEN underlines the good protection given by work clothes/coverall with a penetration rate ranging from 1 to 0.1% in those performing M&A. As for hand exposure, an amount of PEN ranging from 0.1 to about 6500 μg was found, indicating a major contribution of hand to the total actual exposure. This was especially true for those performing re-entry activities, for which a contribution of hand contamination to the actual total exposure exceeded 99%. In this

regard, it should be recognized that gloves were available and recommended, but detailed information about their actual use during re-entry are missing; given the high hand exposure found in these subjects we infer that the use of gloves was spurious or discontinuous. A coherence between the working tasks and dermal exposure was observed, with the highest potential body exposures associated with spraying operations, and the highest hand exposure associated with re-entry.

As for urinary metabolites, PEN-OH and PEN-COOH, concentrations in the range 1.2 to 237 μg/L for PEN-OH and <1 to 54 μg/L for PEN-COOH, were found. These results confirm the previous findings on PEN-OH being much higher than PEN-COOH in human urine, even if the ratio between the two is about 5, lower than 20, the ratio previously reported (Mercadante et al., 2016), and similar to that found after oral administration of PEN to three human volunteers (Sams et al., 2016).

Finding urinary PEN-OH in samples collected before the beginning of the application season suggests that a diffuse contamination from PEN occurs in farmers' working and living environments. Also, in a previous study investigating tebuconazole exposure in agriculture, we observed a contamination of tebuconazole in farmers' workshops and, the presence of monohydroxyl metabolite of tebuconazole, in farmers urine prior to the first use of the season (Fustinoni et al., 2014). Moreover, the presence of PEN and tebuconazole in hair collected from these farmers before the application season further strengths this interpretation (Mercadante et al., 2018).

The concentrations of biomarkers spans over two orders of magnitude, which is consistent with the variability of dermal actual body exposure, but this variability is lower than that of hand exposure and actual total exposure. The lower variability of biomarkers in comparison with dermal exposure is consistent with previous observations (Lin et al., 2005). Moreover, median urinary PEN-OH levels were similar in all study periods, ranging from 8.1 μg/L, in Day 0, to 27.6 μg/L, in

Post 24h-R1. These levels are about one order of magnitude lower than those previously found for the monohydroxy metabolite of tebuconazole, in vineyard farmers (Fustinoni et al., 2014). This is coherent with the lower amount of PEN applied to vineyards in comparison with tebuconazole.

To the best of our knowledge, there was only a previous study ~~on~~ addressing the biomonitoring of PEN exposure (Sams et al., 2016). In this study, 89 urine samples from 48 resident adults and six children were collected after the spray event; in these samples the percentage of detectable PEN-COOH was 19% (limit of detection 0.25 µg/L) and the 95th and maximum levels were 0.9 and 5.1 µg/g creatinine. These levels were slightly higher, but comparable to those obtained in background urine samples of the general population, both within and outwith the spray season (95th percentile 0.8 and 0.6 µg/g creatinine, respectively, maximum 3.3 and 4.8 µg/g creatinine, respectively) (Sams et al., 2016). As expected, these levels, were much lower than those found in the present study for agricultural workers.

The kinetics study shows that a maximum excretion of PEN-OH is found in urine samples collected in the 24 h following the work shift with PEN exposure (Table 3 and Figure 2). In study subjects, absorption of skin deposed pesticide is the major exposure route, and continues over the working time. Considering this scenario, our result is consistent with that of a study in which PEN was orally administered to three human volunteers, and a half time of few hours (3-5 h) was observed for PEN-OH in urine (Sams et al., 2016). Moreover, the kinetics study suggests that the best time for sample collection is the 24 h-interval following exposure, or in case of multiple consecutive exposures, the 24 h-interval following the last exposure. A punctual time for sample collection, similar to what is available for biomarkers with an established application in occupational health and safety protocols (i.e. at the end of shift or prior to the next shift) cannot be specified (ACGIH, 2017); these considerations on the best sampling time are similar to those previously reported for tebuconazole in vineyards (Fustinoni et al., 2014).

Notwithstanding the present study was performed in a real work environment, with several

sources of variability and multiple exposure conditions, positive correlations were observed between the urinary concentration of PEN-OH and dermal exposure (Table 4). The best correlation coefficients were found, firstly between PEN-OH and the actual body exposure, with Pearson's r ranging from 0.386 and 0.562, and secondly between PEN-OH and the actual total exposure, with Pearson's r of 0.279 and 0.310. These results confirm the major relevance of actual exposure in comparison with potential exposure, and suggest a limited contribution of hand exposure in determining internal dose. This could be due to the removal of hand dose with washes and/or a lower dermal absorption of hand in comparison with other parts of the body. All this considering, it is not expected a significant change of the real exposure scenario due to the study protocol.

The present study explored a real work environment, and recruiting subjects was difficult (all together only 22 workers could be involved), due to their spread presence over the rural territory and the limited number of workers in each farm. In addition, several sources of variability and multiple exposure conditions, related to different exposure intensities, application modalities and schedules, including the presence of diffuse contamination of farm and household, contributed to the large inter-subject variability, and to a consequent difficulty in data interpretation. This is a general problem of performing field studies in agriculture.

In conclusion, our study first suggests that biomonitoring may be applied to assess exposure to PEN and, in particular, that the determination of PEN-OH in post-exposure urine is a promising determinant for biomonitoring PEN exposure in agricultural workers.

5. Conflict of interest statement

The authors declare they have no conflicts of interest.

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ABBREVIATIONS

ACGIH American Conference of Governmental Industrial Hygienists

EFSA European Food Safety Authority

EU European Union

EUROSTAT European Union Statistical Office

JMPR Joint Meeting on Pesticide Residues

LOQ limit of quantification

M&A mixing and applications

OECD Organisation for Economic Co-operation and Development

PEN penconazole or (RS)-1-[2-(2,4-dichlorophenyl)pentyl]-1H-1,2,4-triazole

PEN-COOH 4-(2,4-dichlorophenyl)-5-[1,2,4] triazol-1-yl-pentanoic acid or carboxyl-derivative of PEN

PEN-OH 4-(2,4-dichlorophenyl)-5-[1,2,4] triazol-1-yl-pentanol or monohydroxyl-derivative of PEN

R re-entries

TIS turbo ion spray

WS work shift

WHO/FAO World Health Organization/ Food and Agriculture Organization

Figure 1. Penconazole (PEN) metabolic pathway.

Figure 2. Excretion rate of PEN-OH (µg/h) in studied vineyard workers. Blue boxes indicate Pre-WS and M&A, R samples, red boxes indicate the Post 24 h samples; plain boxes are associated with M&A, dashed boxes are associated with R.

Figure 3. Scatter plot, linear regression line and 5- 95 % confidence interval between actual body exposure and the concentration of PEN-OH (µg /L) in Post 24h samples.

Assessment of penconazole exposure in winegrowers using urinary biomarkers

Rosa Mercadante[a], Elisa Polledri[b], Federico Maria Rubino[c], Stefan Mandic-Rajcevic[c], Andrea Vaiani[c], Claudio Colosio[c], Angelo Moretto[d], Silvia Fustinoni[§a,b]

[a] *Department of Clinical Sciences and Community Health, University of Milano, Via S. Barnaba 8, 20122 Milan, Italy*

[b] *Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Via S. Barnaba 8, 20122 Milan, Italy*

[c] *Department of Health Sciences of the University of Milan, Laboratory for Analytical Toxicology and Metabolomics (LaTMA) and International Centre for Rural Health of the University Hospital San Paolo, Via di Rudini 8, Milan, Italy*

[d] *Department of Biomedical and Clinical Sciences, University of Milano, and International Centre for Pesticides and Health Risks Protection (ICPS), "Luigi Sacco" Hospital, Via G.B. Grassi 74, 20157 Milano, Italy*

Corresponding Author:

[§]Authors' correspondence address:

Via S. Barnaba, 8 – 20122 Milan, Italy

phone: +39 02 503 20158

fax: + 39 02 503 20111

e-mail: silvia.fustinoni@unimi.it

Abstract

Penconazole (PEN) is a fungicide used in agriculture. The aim of this work was to evaluate the exposure to PEN in vineyard workers focusing on urinary biomarkers.

Twenty-two agricultural workers were involved in the study; they were investigated during PEN applications and re-entry work, performed for 1-4 consecutive working days, for a total of 42 mixing and applications and 12 re-entries. Potential and actual dermal exposure, including hand exposure, were measured using pads and hand washes. Urine samples were collected starting before the first application, continuing during the work shift, and ending 48 hours after the last shift. The determination of PEN in dermal samples and PEN metabolites in urine was performed by liquid chromatography tandem mass spectrometry.

Dermal potential body exposure and actual total exposure showed median levels ranging from 18 to 3356 and from 21 to 111 µg, respectively. Urinary monohydroxyl-derivative PEN-OH was the most abundant metabolite; its excretion rate peaked within 24 h after the work shift. In this period, median concentrations of PEN-OH and the carboxyl-derivative PEN-COOH ranged from 15.6 to 27.6 µg/L and from 2.5 to 10.2 µg/L, respectively. The concentration of PEN-OH during the work shift, in the 24 h after and in the 25-48 h after the work shift were correlated with actual body and total dermal exposure (Pearson's r from 0.279 to 0.562).

Our results suggest that PEN-OH in the 24 h post-exposure urine is a promising candidate for biomonitoring PEN exposure in agricultural workers.

Keywords: urinary penconazole metabolites; biomonitoring; vineyard workers; dermal exposure; pesticides.

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1. Introduction

Vineyards are extensively present in Italy; of the total utilized agricultural area of about 12.9 million of hectares, which represents 43% of the Italian territory, grapes account for about 5%, with 664.296 hectares (ISTAT, 2010).

Grapevine is subject to the attack of molds, and fungicide pesticides are widely used to control these pests. According to European Union Statistical Office in 2015, about 38.861 tons of fungicides and bactericides were sold in Italy (EUROSTAT, 2015). Among fungicides, conazoles are triazole fungicides that interfere with the biosynthesis of sterols in fungi cell membranes. They are largely used on grapes, and within the class, penconazole (PEN, (RS)-1-[2-(2,4-dichlorophenyl)pentyl]-1H-1,2,4-triazole, CAS number 66246-88-6) is one of the most widely used.

The WHO/FAO Joint Meeting on Pesticide Residues set an acceptable daily intake (ADI) of 0.03 mg/kg body weight/day for PEN, this was (WHO/FAO, 1992) confirmed by the European Food Safety Authority Scientific Report (EFSA, 2008), and recently further reiterated by a new version of the WHO/FAO document (WHO/FAO, 2016). From the toxicological point of view, PEN has low acute toxicity and causes liver toxicity in experimental animals (mice, rats and dogs); PEN is not carcinogenic, nor genotoxic (WHO/FAO, 2016). Reduced mating index and longer gestation duration was observed in rats. In addition, PEN was found to be teratogenic (microphthalmia and hydroencephalus) in rabbits, but not in rats (WHO/FAO, 2016).

According to the harmonized classification and labelling approved by the European Union, PEN is very toxic to aquatic life with long lasting effects (hazard statements H400 and H410), is harmful if swallowed (H302) and is suspected of damaging fertility or the unborn child (H361) (Regulation (EC) No 1272/2008).

Given the use of PEN as a pesticide, and therefore its intentional spread into the environment, agricultural workers, agricultural families and rural residents living in the area surrounding treated

crops, and the general population consuming contaminated crop products and drinking water may be exposed. According to EU, the maximum residue level for PEN on table and wine grapes is 0,2 mg/kg (Commission Regulation (EC) No 149/2008).

Biological monitoring of exposure measures the body burden or the pool of a substance in the body, mostly by quantifying, as biomarker, the substance itself or a suitable bio-transformation product in an easily accessible biological compartment (Angerer et al., 2007; Berode et al., 2011). Recently, the metabolism of PEN in humans was investigated (Mercadante et al., 2016; Sams et al., 2016), showing that the major urinary metabolites are the monohydroxyl-derivative PEN-OH, mostly excreted as glucuronide conjugated, and the carboxyl-derivative PEN-COOH, although with a minor contribution (see Figure 1). Based on this result, we suggested the measurement of PEN-OH concentration in urine, after the hydrolysis of the glucuronide conjugate, for the biological monitoring of PEN exposure (Mercadante et al., 2016).

The aim of this work was to study the behavior of PEN-OH and PEN-COOH as biomarkers of PEN exposure. Twenty-two agricultural workers applying PEN in vineyards were recruited. External exposure was assessed collecting information on the use of PEN and investigating dermal exposure. In fact, dermal exposure was recognized as the main exposure route both during pesticide mixing, loading and application, and during re-entry activities (Aprea et al., 2005; Baldi et al., 2014; Baldi et al., 2006; Flack et al., 2008). PEN metabolites were measured in urine sampled before, during and after the application/re-entry in the treated vineyards.

2. Material and methods

2.1 Study subjects

The field study was performed from May to July 2012. The involved subjects were 22 vineyard workers who sprayed PEN in vineyards and performed post application activity (re-entry). They were recruited from different areas of Lombardy, Italy: ten workers were farm owners of the provinces of Bergamo and Brescia, and twelve workers were agricultural cooperative employees from the province of Sondrio. Agricultural workers mixed, loaded and applied PEN in the form of a diluted oil-in-water emulsion (about 10% w/w) for foliar application on grapes; application was performed with a sideways-spraying tractor-mounted air blast, or spraying upwards with hand-held application equipment. Re-entry workers were involved in monitoring access of grapes to air and sunlight, i.e. cutting buds in excess, removing young plant shoots or excess bunches of grapes, raising or lowering the wires. A total of 42 mixing and applications (M&A) and 12 re-entries (R) were performed. Study subjects worked in vineyards from 1 to up to 4 consecutive daily work shifts.

The research team administered the questionnaire, supervised fieldwork and collected data on personal characteristics, such as age, height, body weight, and occupational information useful to explore determinants of exposure. The latter included: the name and brand of the formulation; the concentration of PEN in the formulation; the size of the treated area; the quantity of formulation applied per area; the work shift duration; the application equipment and techniques; any maintenance of the machinery; the type of clothing and personal protective equipment worn during the work.

The study was performed in the context of the risk evaluation, according to Italian law for health and safety at the workplace (Decreto Legislativo 9 aprile 2008). A field staff that included technical and sanitary professionals performed the study. Each study subject read, understood and

signed the informed consent form, also with reference to yielding urine samples for biological monitoring.

2.2 Dermal sampling

Dermal exposure was assessed by the use of pads and the coordinated collection of the hand wash liquid. Body exposure was assessed using square 0.01 m^2 lab-made pads prepared with filter paper (Whatman no.1 filter paper, Prodotti Gianni, Milan) as previously described (Mandic-Rajcevic et al., 2018; OECD, 1997; Rubino et al., 2012). Pads were applied on six representative body districts, namely: right upper limb (arm + right forearm), left upper limb (arm + left forearm), front trunk (thorax + abdomen), rear trunk (back + loin), right lower limb (thigh + right shin), and left lower limb (thigh + left shin). Twelve pads were applied for each subject for the whole work shift, six of which above clothes and six under clothes; for each body district two different positions were selected to avoid superimposition. At the end of the work shift, each dermal pad was detached from clothes/skin, coded and kept in the dark at room temperature. In total, more than seven hundreds dermal samples were collected. To assess hand exposure, a modification of the "Hand rinse method" reported in the OECD guidelines (OECD, 1997) was used; in particular 100 ml of a solution of isopropyl alcohol in water (25% v/v) were used; this solution was chosen for its optimum solvent property and the low toxicity. In particular, a member of the research staff slowly poured the washing solution over the cupped hands of the worker for about 30-60 s while the worker rubbed his hands together. Hand wash liquid was collected in a basin underneath and then poured in a dark coded glass bottle, and stored at room temperature. The recovery of PEN during this operation was over 90%. The wash was performed at the end of the work with pesticides and/or at any other work break during the shift, according to subject's need. About 130 hand wash samples were collected. All samples were delivered to the laboratory within one week of collection; once in the laboratory, samples were kept at – 20 °C until analysis, that was performed within 6 months.

Pads on clothes were used to estimate the potential body exposure, defined as the amount of pesticide deposited on work clothes and/or coveralls. Pads under the clothes were used to estimate actual body exposure, defined as the amount of pesticide deposited on worker's skin, and that is the amount of pesticide able to reach the uncovered skin and available for absorption. Finally, actual total exposure was calculated as the sum of actual body exposure *plus* hand exposure.

2.3 Urine collection for biological monitoring

Urine samples were self-collected by workers during the study period. To this aim, workers were provided with instructions and a sampling kit consisting of sampling bottles and a collection sheet to record sampling time. Each subject was asked to collect pooled urine sample for the following periods:

- during the 24 h before the first work shift with the use of PEN (Pre-WS) (Day 0);
- during the work shift/s with PEN exposure due to M&A and/or R (Day 1 to 4);
- after M&A and R work shift/s, for 24 h or for a shorter period, for those who performed multiple consecutive work shifts (Post 24h/Post 24h-R) (Day 1 to 4);
- for additional 24 h after the Post 24h sample, until 48h after the last exposure (Post 25-48h) (Last day).

Samples were kept at room temperature, 20 - 25 °C, and retrieved, by study staff, at the end of each day. Urine volume was measured (± 5 mL) and an aliquot was taken and cooled to 4 °C for the analysis. Aliquots were delivered to the laboratory within 72 h after the end of collection. In the laboratory, aliquots were stored at -20 °C until analysis, which occurred within 6 months. The number of urine samples collected by each subject varied, based on the number of consecutive working days; all together 148 urine samples were obtained.

2.4 Measurement of PEN and PEN metabolites

For the preparation of standard solutions, PEN (purity 98.7%, Sigma-Aldrich, Milan, Italy), PEN-OH, and PEN-COOH (purity 98% for both, kindly donated by Syngenta, UK) were used. For

the preparation of the internal standard solution (IS), penconazole-d7 (98 atom % D, PEN-d7, Sigma-Aldrich, Milan, Italy) was used.

The measurement of PEN on dermal samples (pads) was performed after the desorption overnight at room temperature with 8 mL of an aqueous solution of 25% isopropyl alcohol in the presence of penconazole-d_7 (98 atom % D, PEN-d_7, Sigma-Aldrich, Milan, Italy) as internal standard. An aliquot of each solution was analyzed by high performance liquid chromatography (Surveyor, Thermo Scientific, Rodano, Italy) equipped with a Betasil C18 column (150 mm length, 2.1 mm internal diameter and 5 µm particle size; Thermo Scientific, Rodano, Italy) kept at room temperature, using an isocratic mixture of aqueous formic acid (0.5%) and methanol (30:70) at 250 µl/min as eluent. The liquid chromatography was interfaced with a LC-MS/MS (TSQ Quantum Access, Thermo Scientific, Rodano, Italy) equipped with a heated-electro spray ionization source. The extraction efficiency was about 98%. The intra- and inter-day precision of the method, assessed as a percent coefficient of variation, was less than 10%, the accuracy was 95-103%, and the limits of quantification (LOQ) was 1 µg/L. Field blanks, included to check the possible contamination of sampling material during the field study, showed no contamination.

The measurement of PEN metabolites in urine was performed as previously described (Mercadante et al., 2016). Briefly, urine samples were thawed at room temperature, added with a penconazole-d_7 solution, as internal standard, and β-glucuronidase hydrolysis solution. Hydrolysis was performed at 40 °C overnight. Analyses were performed as previously described (Mercadante et al., 2016), using a liquid chromatography-triple quadruple mass spectrometry (4500 System, SCIEX, Milano, Italy) equipped with a turbo ion spray (TIS) source interfaced with a binary pump (Shimadzu, Milano, Italy). The chromatographic separation was performed with a Hypersil Gold PFP column (50 mm length, 2.1 mm internal diameter, and 3 µm particle size; Thermo Scientific, Rodano, Italy) and with 0.1% aqueous formic acid (eluent A) and methanol (eluent B), at a flow rate of 500 µl/min. The analytes were detected in the positive ion mode and quantified based on

single reaction monitoring. The intra- and inter-day precision of the method, assessed as a percent coefficient of variation, was less than 10%, accuracy was between 100 and 108%, and the limit of quantification (LOQ) was 2 µg/L for PEN and 1 µg/L for both PEN-OH and PEN-COOH. For quality controls (QC), as no certified material was purchasable, in-house quality control solutions were prepared and analyzed together with samples in any analytical sequence. A typical analytical sequence consisted of analyzing calibration curve samples, followed by ten prepared unknown samples analyzed along with one duplicate sample, one low-QC, and one high-QC sample, followed by a second set of calibration curve samples (Mercadante et al., 2016). The throughput was about 50 samples/day.

Urinary creatinine was determined using Jaffe's colorimetric method (Kroll et al., 1986).

2.5 Data processing and statistical analysis

Calculations to estimate dermal exposure (µg) were performed as described elsewhere (Mandic-Rajcevic et al., 2018; Mandic-Rajcevic et al., 2015).

Briefly, the percentages of body surface represented by each pad were calculated using the Mosteller formula (Mosteller, 1987), considering the proportions of body areas of a normal healthy male. The potential body exposure was calculated as the sum of regional exposures which were measured by external pads (on the clothes), taking into account the surface of the pad and the body region represented by each pad. The actual body exposure was calculated as the sum of regional exposures calculated from the amount of PEN measured in the skin pads multiplied by the surface of each region represented by the pads. The actual total exposure was calculated summing actual body exposure with the amount of PEN on hand.

To describe the kinetics of PEN-OH, data were expressed as excretion rate (µg/h), taking into consideration urine volume and the duration of urine collection.

In order to ascertain whether the workers collected all urine voids, since no information on incomplete urine collection was reported, we calculated the expected daily amount of urinary

creatinine $Creat_{exp}$, according to the formula (Knuiman et al., 1986):

$Creat_{exp}$ (g/day) = body weight (kg) X 24 (g/(day x kg))

From the ratio between measured and expected daily amount of urinary creatinine, the percentage of incomplete urine collection was estimated (results reported in Table 1). This percentage is however affected by a large degree of variability and only when it falls outside the range 60 – 140%, urine sample should be considered unreliable (Joossens JV, 1984).

Finally the level of PEN-OH and PEN-COOH were also expressed in µg/g creatinine.

Statistical analysis was performed using SPSS 25.0 for Windows (IBM SPSS Statistics). Values below the LOQ were assigned a value equal to 1/2 LOQ. For the descriptive analysis median, minimum and maximum values of the distribution were calculated; moreover, for PEN-OH and PEN-COOH the percentage of quantified samples was added.

The correlation between dermal exposure and the urinary concentration of PEN-OH in the different periods was evaluated using Pearson's correlation performed on log-transformed variables. Due to the limited number of available observations, they were grouped based on the time frame. M&A1, M&A2, M&A3+R1, R2 samples were pooled and considered all part of the M&A+R group; Post 24h-1, Post 24h-2, Post 24h-3, Post 24h-R1, and Post 24h-R2 samples were pooled and considered in the Post 24h group. Pooled data were considered as independent observations.

3. Results

3.1 Study subjects

In Table 1, a summary of personal characteristics of study subjects and some information on the work shifts with exposure to PEN is reported. All subjects, but one, were males, with median age, height and weight of 48 years, 175 cm and 78 kg, respectively. During urine collection workers excreted a median creatinine amount of 1.3 g/day (from 0.9 to 2.4 g/day), and this was estimated to be 71% of the expected amount; 5 out of 22 subjects had median creatinine level below 60%, and their urine samples should be considered incomplete. In particular 4 out of 5 subjects had median values between 50 and 60 % (almost acceptable) and just one subject had very low creatinine level, that was 38% of the theoretical value.

Eight workers applied PEN during one work shift, two workers applied PEN for two consecutive work shifts, and six workers applied PEN for three consecutive work shifts. Moreover, six subjects applied PEN for two consecutive work shifts and performed re-entry activities for other two consecutive work shifts. In general, applicators performed different tasks: prepared the fungicide mixture, loaded the tank of the sprayer, either tractor-mounted or hand-held, sprayed the pesticide, and cleaned the equipment. Re-entry growers were mostly involved in monitoring access of grapes to air and sunlight, i.e. cutting buds in excess, removing young plant shoots or excess bunches of grapes, raising or lowering the wires, cutting excess branches (Baldi et al., 2014). The quantity of PEN sprayed ranged from 26 to 1500 g, the treated area ranged from 0.4 to 11 ha, and the work shift duration ranged from 1 to 12 h.

3.2 Dermal exposure

The results of dermal exposure to PEN are reported in Table 2. The potential body exposure during the different work shifts ranged 4.2 to 9197.3 µg during M&A, and from 6.2 to 56.0 µg during R. A tiny amount of PEN passed through the work clothes/coverall, with actual body exposure ranging from 0.9 to 526.7 µg during M&A, and from 0.8 to 4.6 µg during R. Median

exposure on hands ranged from 12.1 to 109.3 µg, with maximum levels up to 6482.4 µg during M&A3. Body and hand exposure contributed differently to the actual total exposure, accordingly to work tasks. During M&A, potential body exposure was quite high, but the corresponding actual body exposure was from 1/100 to 1/1000 lower, showing that work clothes/coveralls afford a good protection. For workers performing M&A, hand exposure and actual body exposure similarly contributed to actual total exposure. During re-entry activities, potential body exposure was low, and consequently actual body exposure was very low; conversely, hand exposure was about two orders of magnitude higher, so that hand exposure resulted to be the major contributor of actual total dermal exposure. Total exposures in those performing re-entry activities was about 2-fold higher than in those performing M&A.

3.3 Biological monitoring and excretion rate

The duration of urine collection ranged from 4.5 to 31.5 h, with a minimum value during work shifts and a maximum on Day 0; urine volume ranged from 117 to 2842 ml. Although a general coherence between sample volume and sampling duration was found, a more detailed analysis performed using the daily amount of urinary creatinine (Table 1) lead us to conclude that the majority of study subjects properly collected urine samples.

In Table 3, the concentration of PEN metabolites in urine (expressed as µg/L and µg/g crea), and the excretion rate of PEN-OH referring to samples obtained during both the work shift and the post working period, were reported for each investigated day. The median concentration of PEN-OH ranged from 8.0 to 27.6 µg/L, with a maximum concentration of 237 µg/L in Post 25-48h. Considering creatinine excretions, the median concentration of PEN-OH ranged from 8.2 to 20.2 µg/g crea, with a maximum concentration of 301 µg/g crea in Post 25-48h. PEN-OH was quantified in all samples, including during Day 0, although workers declared that they had not used PEN in the previous part of the application season.

The median concentration of PEN-COOH ranged from <1 to 10.2 µg/L, with a maximum

concentration of 54.1 µg/L in Post 24h-3. PEN-COOH was quantified in 43 to 100% of samples, with a minimum percentage during Day 0. Considering creatinine excretions, the median concentration of PEN-COOH ranged from 0.9 to 12.6 µg/g creat, with a maximum concentration of 64.8 µg/g creatinine in Post 25-48h.

Median ratio between PEN-OH and PEN-COOH was about 5 (3.8 - 8.8), highlighting that in humans PEN is mostly metabolized and excreted as PEN-OH, both as glucuronide conjugated and in the free form (see Figure 1) (Mercadante et al., 2016; Sams et al., 2016).

Median PEN-OH excretion rate ranged from 0.37 to 1.19 µg/h, with a maximum value of 7.36 µg/h in Post 25-48h. In the box-plot graph of Figure 2, the excretion rates are reported. An increase in the excretion rate was observed comparing PEN-OH rate in samples collected during the work shift (M&A/R) and in the 24 h after the work shift (Post 24h). Considering consecutive days, a trend toward increasing values was observed, passing from Day 0 to Day 3, suggesting an accumulation of PEN-OH following consecutive exposures. Starting from Day 4, a decrease in the excretion rate was observed, with a minimum value of 0.48 µg/h in the last day.

Comparing PEN-OH concentration during M&A vs. R, no large difference was found. This was unexpected as actual total exposure was almost double in those who performed re-entry than in those who performed M&A. This suggests that hand exposure was less efficient than body exposure in determining internal dose of PEN and/or that additional exposure via inhalation could have contributed to the internal dose for M&A.

3.4 Correlation between dermal exposure and urinary PEN-OH

In Table 4, Pearson's correlation coefficients between dermal exposure and the concentration of PEN-OH in the different time frames are reported. Considering PEN-OH (µg/L), significant correlations were found between actual body exposure, for all time frames, with Pearson's r ranging from 0.386 to 0.562 and p values ≤ 0.01. Significant correlations were also found between actual total exposure and PEN-OH during M&A+R and Post 24h samples, with a

Pearson's r of 0.279 and 0.310 respectively, and p values ≤ 0.05. Similar or better correlations were obtained when PEN-OH was adjusted for creatinine,

The scatter plot, linear regression line and confidence interval between actual body exposure and PEN-OH in the Post 24h, are reported in Figure 3. On the contrary, no significant correlation was found between PEN-OH and potential body exposure or hand exposure.

4. Discussion

Following a previous study in which PEN-OH, as glucuronide conjugate and in the free form, was identified as the major metabolite of PEN in human urine, this work shows that total PEN-OH excreted in urine is a useful biomarker of exposure to PEN. Moreover, it identifies the 24 h post exposure period as the best time for sample collection.

This study is very notable for many aspects. First, the study protocol entails simultaneous collection of dermal and urine samples, and results allow to correlate dermal exposure and urinary PEN-OH. Second, consecutive urine samples were collected starting before the first application of the season and continuing until 48 h after the end of the last work shift with exposure to PEN. This allowed investigating the formation of the main metabolites of PEN, and their respective kinetics of urinary elimination. Third, the study protocol followed workers across several days, where activities included consecutive spraying days and re-entry, and resting periods. This allowed comparing exposures associated to different tasks in agriculture.

As shown in Table 1, 22 study subjects experienced different exposure patterns, such as one to three consecutive application shifts, and the combination of applications and re-entries. This, together with the different application procedures, the variable amount of PEN applied, and the different working times, yield a wide spectrum of exposures, as reflected by the large variability of dermal potential body exposure that spanned over three orders of magnitude, from few μg up to almost 10 mg. As for actual body exposure, a reduction of PEN and its availability for absorption is observed, with values ranging from below 1 μg to 500 μg, therefore spanning over two orders of magnitude. The low amount of PEN underlines the good protection given by work clothes/coverall with a penetration rate ranging from 1 to 0.1% in those performing M&A. As for hand exposure, an amount of PEN ranging from 0.1 to about 6500 μg was found, indicating a major contribution of hand to the total actual exposure. This was especially true for those performing re-entry activities, for which a contribution of hand contamination to the actual total exposure exceeded 99%. In this

regard, it should be recognized that gloves were available and recommended, but detailed information about their actual use during re-entry are missing; given the high hand exposure found in these subjects we infer that the use of gloves was spurious or discontinuous. A coherence between the working tasks and dermal exposure was observed, with the highest potential body exposures associated with spraying operations, and the highest hand exposure associated with re-entry.

As for urinary metabolites, PEN-OH and PEN-COOH, concentrations in the range 1.2 to 237 μg/L for PEN-OH and <1 to 54 μg/L for PEN-COOH, were found. These results confirm the previous findings on PEN-OH being much higher than PEN-COOH in human urine, even if the ratio between the two is about 5, lower than 20, the ratio previously reported (Mercadante et al., 2016), and similar to that found after oral administration of PEN to three human volunteers (Sams et al., 2016).

Finding urinary PEN-OH in samples collected before the beginning of the application season suggests that a diffuse contamination from PEN occurs in farmers' working and living environments. Also, in a previous study investigating tebuconazole exposure in agriculture, we observed a contamination of tebuconazole in farmers' workshops and, the presence of monohydroxyl metabolite of tebuconazole, in farmers urine prior to the first use of the season (Fustinoni et al., 2014). Moreover, the presence of PEN and tebuconazole in hair collected from these farmers before the application season further strengths this interpretation (Mercadante et al., 2018).

The concentrations of biomarkers spans over two orders of magnitude, which is consistent with the variability of dermal actual body exposure, but this variability is lower than that of hand exposure and actual total exposure. The lower variability of biomarkers in comparison with dermal exposure is consistent with previous observations (Lin et al., 2005). Moreover, median urinary PEN-OH levels were similar in all study periods, ranging from 8.1 μg/L, in Day 0, to 27.6 μg/L, in

Post 24h-R1. These levels are about one order of magnitude lower than those previously found for the monohydroxy metabolite of tebuconazole, in vineyard farmers (Fustinoni et al., 2014). This is coherent with the lower amount of PEN applied to vineyards in comparison with tebuconazole.

To the best of our knowledge, there was only a previous study addressing the biomonitoring of PEN exposure (Sams et al., 2016). In this study, 89 urine samples from 48 resident adults and six children were collected after the spray event; in these samples the percentage of detectable PEN-COOH was 19% (limit of detection 0.25 µg/L) and the 95th and maximum levels were 0.9 and 5.1 µg/g creatinine. These levels were slightly higher, but comparable to those obtained in background urine samples of the general population, both within and outwith the spray season (95th percentile 0.8 and 0.6 µg/g creatinine, respectively, maximum 3.3 and 4.8 µg/g creatinine, respectively) (Sams et al., 2016). As expected, these levels, were much lower than those found in the present study for agricultural workers.

The kinetics study shows that a maximum excretion of PEN-OH is found in urine samples collected in the 24 h following the work shift with PEN exposure (Table 3 and Figure 2). In study subjects, absorption of skin deposed pesticide is the major exposure route, and continues over the working time. Considering this scenario, our result is consistent with that of a study in which PEN was orally administered to three human volunteers, and a half time of few hours (3-5 h) was observed for PEN-OH in urine (Sams et al., 2016). Moreover, the kinetics study suggests that the best time for sample collection is the 24 h-interval following exposure, or in case of multiple consecutive exposures, the 24 h-interval following the last exposure. A punctual time for sample collection, similar to what is available for biomarkers with an established application in occupational health and safety protocols (i.e. at the end of shift or prior to the next shift) cannot be specified (ACGIH, 2017); these considerations on the best sampling time are similar to those previously reported for tebuconazole in vineyards (Fustinoni et al., 2014).

Notwithstanding the present study was performed in a real work environment, with several

sources of variability and multiple exposure conditions, positive correlations were observed between the urinary concentration of PEN-OH and dermal exposure (Table 4). The best correlation coefficients were found, firstly between PEN-OH and the actual body exposure, with Pearson's r ranging from 0.386 and 0.562, and secondly between PEN-OH and the actual total exposure, with Pearson's r of 0.279 and 0.310. These results confirm the major relevance of actual exposure in comparison with potential exposure, and suggest a limited contribution of hand exposure in determining internal dose. This could be due to the removal of hand dose with washes and/or a lower dermal absorption of hand in comparison with other parts of the body. All this considering, it is not expected a significant change of the real exposure scenario due to the study protocol.

The present study explored a real work environment, and recruiting subjects was difficult (all together only 22 workers could be involved), due to their spread presence over the rural territory and the limited number of workers in each farm. In addition, several sources of variability and multiple exposure conditions, related to different exposure intensities, application modalities and schedules, including the presence of diffuse contamination of farm and household, contributed to the large inter-subject variability, and to a consequent difficulty in data interpretation. This is a general problem of performing field studies in agriculture.

In conclusion, our study first suggests that biomonitoring may be applied to assess exposure to PEN and, in particular, that the determination of PEN-OH in post-exposure urine is a promising determinant for biomonitoring PEN exposure in agricultural workers.

5. Conflict of interest statement

The authors declare they have no conflicts of interest.

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ABBREVIATIONS

ACGIH American Conference of Governmental Industrial Hygienists

EFSA European Food Safety Authority

EU European Union

EUROSTAT European Union Statistical Office

JMPR Joint Meeting on Pesticide Residues

LOQ limit of quantification

M&A mixing and applications

OECD Organisation for Economic Co-operation and Development

PEN penconazole or (RS)-1-[2-(2,4-dichlorophenyl)pentyl]-1H-1,2,4-triazole

PEN-COOH 4-(2,4-dichlorophenyl)-5-[1,2,4] triazol-1-yl-pentanoic acid or carboxyl-derivative of PEN

PEN-OH 4-(2,4-dichlorophenyl)-5-[1,2,4] triazol-1-yl-pentanol or monohydroxyl-derivative of PEN

R re-entries

TIS turbo ion spray

WS work shift

WHO/FAO World Health Organization/ Food and Agriculture Organization

Figure 1. Penconazole (PEN) metabolic pathway.

Figure 2. Excretion rate of PEN-OH (µg/h) in studied vineyard workers. Blue boxes indicate Pre-WS and M&A, R samples, red boxes indicate the Post 24 h samples; plain boxes are associated with M&A, dashed boxes are associated with R.

Figure 3. Scatter plot, linear regression line and 5- 95 % confidence interval between actual body exposure and the concentration of PEN-OH (µg /L) in Post 24h samples.

Table

Table 1. Main personal characteristics of study farmers and information on the work shifts with exposure to penconazole (PEN).

		Statistics	All subjects	Investigated work shifts			
				Day 1	Day 2	Day 3	Day 4
Study subjects		n	22	22	14	12	6
Gender	M	n	21	-	-	-	-
	F	n	1	-	-	-	-
Age (year)		Median (min-max)	48 (22 – 59)	-	-	-	-
Height (cm)		Median (min-max)	175 (162 – 187)	-	-	-	-
Weight (kg)		Median (min-max)	78 (55 – 113)	-	-	-	-
Creatinine (g/die)		Median (min-max)	1.3 (0.9 – 2.4)	-	-	-	-
Expected Creatinine (g/die)		Median (min-max)	1.9 (1.3 – 2.7)	-	-	-	-
Creatinine ratio (%)		Median (min-max)	71 (38 – 106)	-	-	-	-
Job tasks	M&A	n	42	22	14	6	0
	R	n	12	0	0	6	6
Amount of PEN applied (g)		median (min-max)	90 (26 - 1500)	90 (36 - 1500)	210 (35 – 900)	13 (26 – 75)	0
Work shift duration (h)		median (min-max)	8 (1-12)	8 (1– 12)	8 (6 – 8)	8	6

A&M = mixing & application
R = re-entry activities

Table 2. Summary of dermal exposure to PEN in study subjects.

	statistics	Day 1	Day 2	Day 3		Day 4
		M&A1	M&A2	M&A3	R1	R2
Potential body exposure (µg)	Median Min-Max	3356.5 8.2-9197.3	2361.6 4.2-6401.5	2783.3 443.4-6878.8	18.9 6.2-48.2	18.0 8.4-56.0
Actual body exposure (µg)	Median Min-Max	3.3 0.9-526.7	30.9 1.9-128.7	10.4 2.1-64.2	1.3 1.0-4.6	1.3 0.8-2.4
Hands (µg)	Median Min-Max	12.1 0.1-282.3	12.1 0.4-76.2	42.8 4.3-6482.4	109.3 91.0-306.2	109.6 40.3-346.2
Actual total exposure (µg)	Median Min-Max	20.6 1.6-573.2	46.6 4.1-286.2	78.7 10.4-6522.9	111.2 94.2-307.5	110.5 41.8-348.2

Table 3. Summary of urinary samples, concentration of PEN-OH and PEN-COOH and PEN-OH excretion rate.

		statistics	**Day 0**	**Day 1**		**Day 2**		**Day 3**				**Day 4**		**Last day**
			Pre-WS	M&A1	Post 24h-1	M&A2	Post 24h-2	M&A3	R1	Post 24h-3	Post 24h-R1	R2	Post 24h-R2	Post 25-48h
		N samples	21	19	22	14	14	6	6	6	6	6	6	22
Length of time frame (h)		Median Min-Max	23.5 11.5-31.5	10.0 4.5-12.5	15.3 6.5-26.5	7.5 4.5-9.5	17.5 12.0-25.0	7.5 6.0-11.0	9.8 9.0-10.5	25.5 24.0-27.0	14.0 13.5-15.0	9.0 4.5-10.5	24.0 23.5-29.0	24.0 22.5-25.5
Urine volume (ml)		Median Min-Max	1242 352-2842	440 117-758	752 178-2165	431 177-709	716 356-1865	376 152-651	673 281-872	895 164-1437	821 408-2098	666 373-1258	1469 731-2406	1349 273-2489
PEN-OH	**µg/L**	Median Min-Max	8.1 1.4-34.4	11.8 3.1-30.7	16.7 1.4-128.0	15.0 1.7-107.0	18.8 1.3-150.0	13.5 3.5-112.0	13.8 1.4-47.6	25.2 11.4-59.3	27.6 2.9-73.3	8.0 1.1-13.4	15.6 5.4-42.7	9.4 1.2-237.0
	µg/g crea	Median Min-Max	8.2 2.2-28.0	9.8 1.2-73.7	13.6 1.2-94.1	14.2 4.3-60.0	16.5 0.8-98.1	14.2 2.9-71.4	13.9 1.2-47.4	15.3 7.4-70.8	19.2 4.7-35.5	9.2 1.2-21.0	20.2 4.0-34.3	10.5 1.8-301.6
	%>LOQ		100	100	100	100	100	100	100	100	100	100	100	100
PEN-COOH	**µg/L**	Median Min-Max	<1.0 <1.0-16.5	1.9 <1.0-20.9	2.9 <1.0-40.3	2.6 <1.0-20.5	5.6 <1.0-36.6	3.1 2.3-23.6	2.2 <1.0-16.0	10.2 3.1-54.1	5.6 <1.0-41.2	2.0 <1.0-9.9	2.5 <1.0-9.6	1.8 <1.0-50.9
	µg/g crea	Median Min-Max	1.0 (0.3-37.8)	0.9 (0.4-22.6)	2.2 (0.4-26.9)	2.3 (0.5-31.7)	4.4 (0.3-24.0)	3.4 (1.5-15.1)	2.6 (0.4-31.5)	12.6 (1.3-36.2)	3.0 (0.8-33.2)	2.4 (0.6-19.0)	2.7 (0.9-19.3)	2.1 (0.5-64.8)
	%>LOQ		43	63	77	79	86	100	83	100	83	77	83	68
PEN-OH/PEN-COOH		Median Min-Max	8.6 0.4-39.6	8.8 0.4-48.0	4.3 0.5-31.8	5.0 1.7-31.4	4.6 0.2-14.0	4.2 1.5-8.0	5.3 1.5-8.4	4.3 0.4-5.6	5.7 1.1-8.4	3.8 1.1-5.1	4.6 1.8-15.2	4.4 0.2-32.9
PEN-OH excretion rate (µg/h)		Median Min-Max	0.37 0.07 -1.59	0.51 0.16 -1.88	0.61 0.11-1.09	0.77 0.21-1.06	0.78 0.06-1.36	0.73 0.07-1.15	1.06 0.08-2.00	0.46 0.22-1.18	1.19 0.41-1.36	0.47 0.10 -1.11	0.96 0.32-0.96	0.48 0.09 -7.36

Table 4. Pearson's correlations (r) and p values between dermal exposure and urinary PEN-OH (µg/L and µg/g creat) in different time frames.

	Statistics	PEN-OH (µg/L)			PEN-OH (µg/g crea)		
		M&A + R	Post 24h	Post 25-48h	M&A + R	Post 24h	Post 25-48h
	N data	51	54	22	51	54	22
Potential body exposure (µg)	Pearson's r (p value)	0.273 (0.053)	0.145 (0.295)	0.331 (0.132)	0.207 (0.145)	0.079 (0.573)	0.220 (0.326)
Actual body exposure (µg)		0.488 (<0.001)	0.386 (0.004)	0.562 (0.006)	0.381 (0.006)	0.364 (0.007)	0.572 (0.005)
Hands (µg)		0.123 (0.390)	0.192 (0.165)	0.238 (0.287)	0.163 (0.254)	0.224 (0.106)	0.352 (0.108)
Actual total exposure (µg)		0.279 (0.047)	0.310 (0.023)	0.310 (0.160)	0.284 (0.043)	0.324 (0.018)	0.424 (0.049)

Figure

Figure 1. Penconazole (PEN) metabolic pathway.

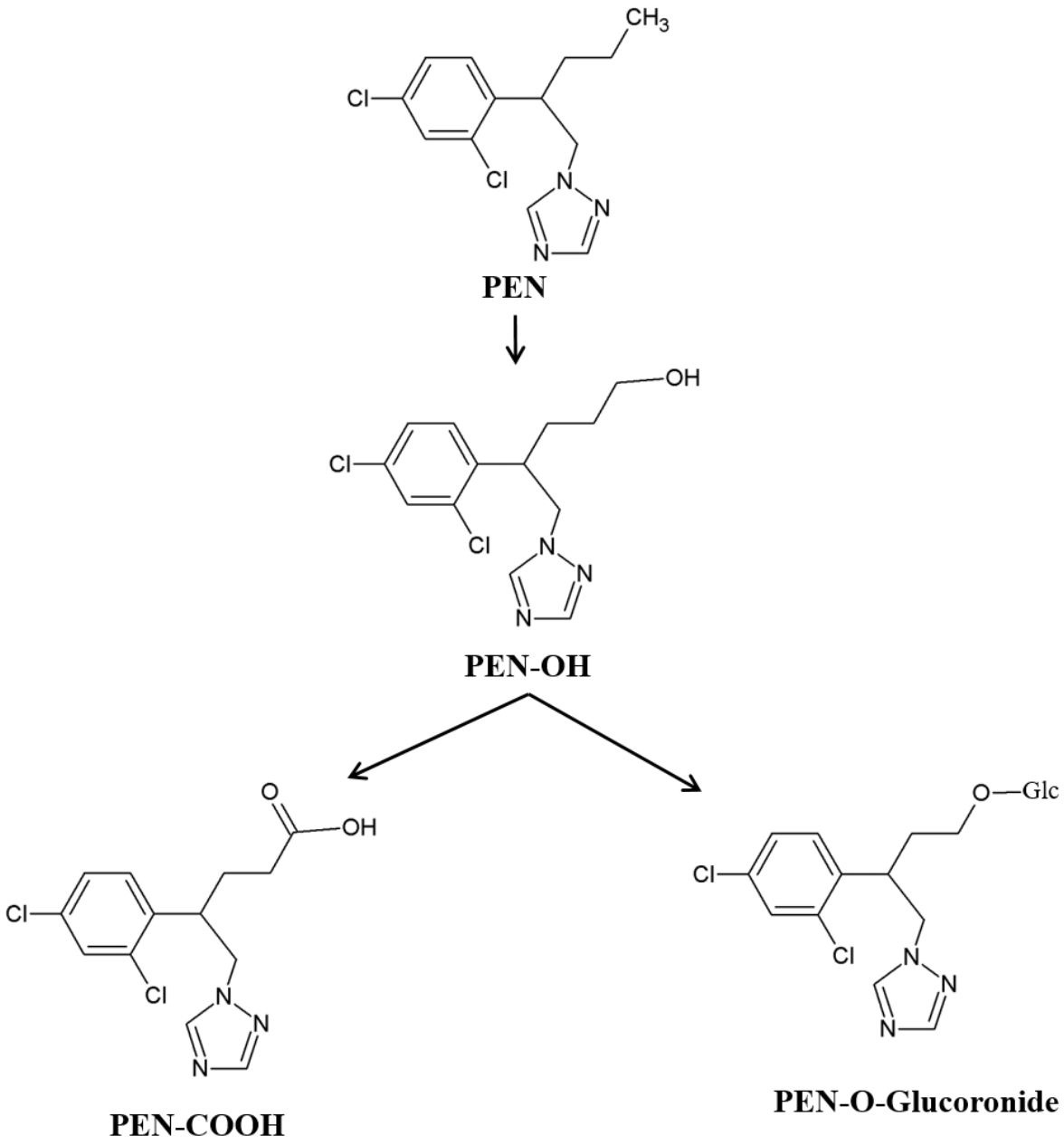

Figure 2. Excretion rate of PEN-OH (µg/h) in study subjects.

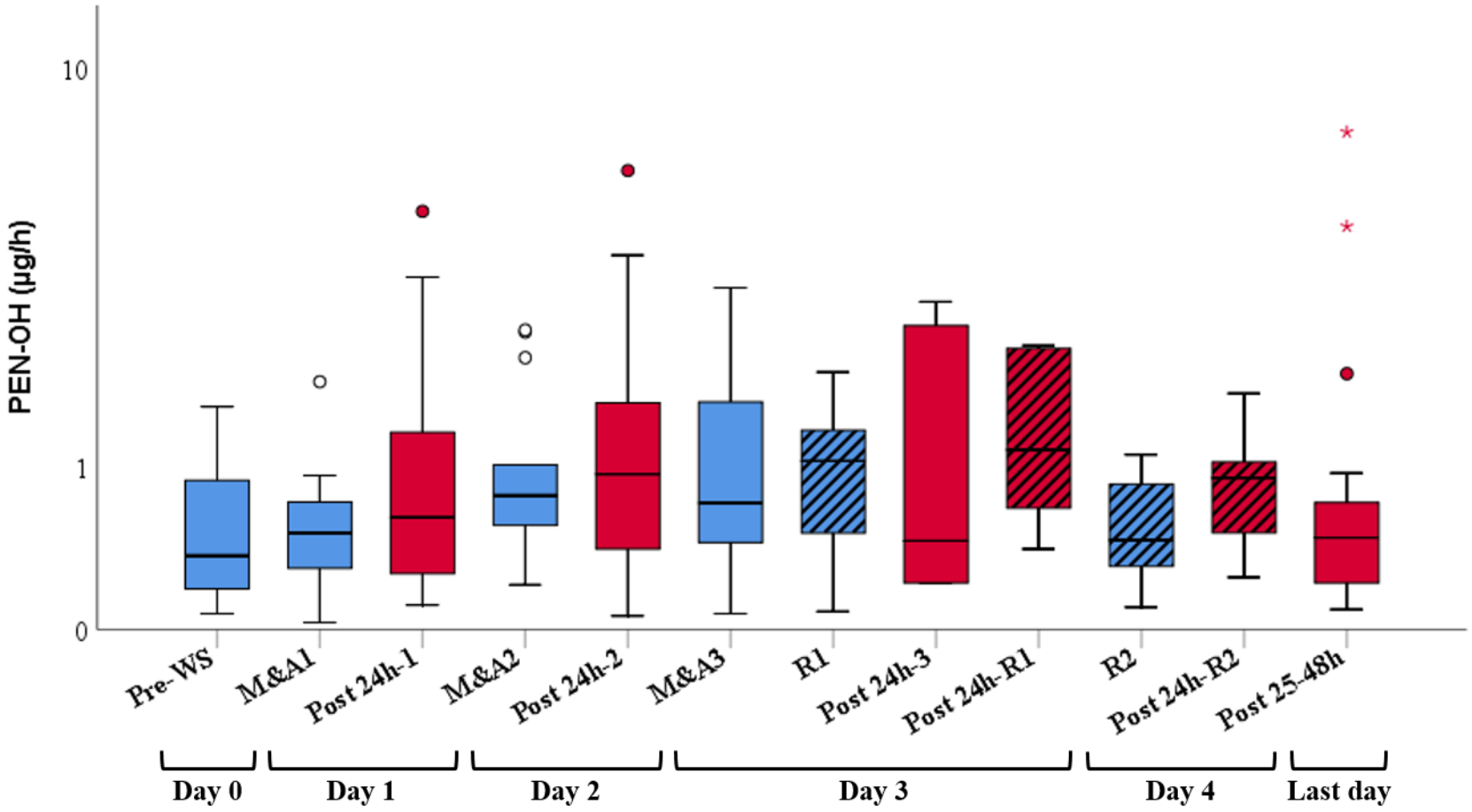

Figure 3. Scatter plot, linear regression line and 5 - 95 % confidence interval between actual body exposure and concentration of PEN-OH (µg/L) in Post 24h samples.