Urine collection methods for non-toilet-trained children in biological monitoring studies: Validation of a disposable diaper for characterization of tebuconazole exposure

A. Oerlemans\textsuperscript{a,b}, M.F.P. van Dael\textsuperscript{b}, R.C.H. Vermeulen\textsuperscript{b}, F.G.M. Russel\textsuperscript{c}, P.T.J. Scheepers\textsuperscript{a}

\textsuperscript{a} Radboud Institute for Health Sciences, Radboud University Medical Center, Nijmegen, the Netherlands
\textsuperscript{b} Institute for Risk Assessment Sciences, University Utrecht, Utrecht, the Netherlands
\textsuperscript{c} Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, Nijmegen, the Netherlands

ARTICLE INFO

Keywords:

Urine collection
Biomonitoring
Non-toilet-trained children
Disposable diaper
Pesticide exposure
Tebuconazole
Creatinine

ABSTRACT

Young children differ from adults in their exposure and susceptibility to environmental chemicals (e.g. pesticides) because of various factors such as behavior, diet and physiology. Their heightened vulnerability to environmental stressors makes it important to obtain appropriate urine samples for exposure characterization. However, collecting urine from non-toilet-trained children has been shown to be methodologically and practically challenging. Four urine collection approaches were tested: a disposable diaper, a urine bag, a collection pad and the clean catch. The success rate and the user rating of each method was evaluated. The success rates were 67\%, 21\%, 17\% and 4\% for the disposable diaper, urine bag, collection pad and clean catch, respectively. The average user ratings on a 0–10 (0 = inconvenient, 10 = convenient) scale were 9.0, 4.7, 7.3 and 2.5, respectively. Subsequently, the best rated method, the disposable polyacrylate diaper was tested with hydroxy-tebuconazole as an exposure biomarker for the fungicide tebuconazole and creatinine for urine density adjustment. After LC-MS/MS analysis, the recoveries of hydroxy-tebuconazole in the range of 0.05–25 ng/mL were on average 106\%, and for creatinine 87\%. Precisions (relative standard deviation) were for both 3\%. The overall procedure including collection and extraction was assessed, resulting in three out of seven positive samples.

Based on this study, the disposable diaper is a suitable method for urine collection of non-toilet-trained children for biomonitoring of tebuconazole. This method can serve as a basis for extension to other substances of interest.

1. Introduction

Infants and young children differ from adults in their exposure and susceptibility to environmental chemicals due to various factors such as biometry, physiology, and behavior. Furthermore, children between 0–3 yr. old are rarely included in biomonitoring studies and thus the information on the exposure of chemical compounds of this group is very limited. This stresses the importance to obtain appropriate data for exposure assessment, specifically for very young children which are at their developing age (Needham and Sexton, 2000). The omnipresence of pesticides, the products of environmental degradation, and the potential exposure to pesticides is an increasing public health concern. The actual exposure and the potential adverse health effects of pesticides are largely unknown. Previous studies suggest that pesticide exposure could be associated with neurodevelopmental impairment, immunologic abnormalities, and end-stage renal disease (Gonzalez-Alzaga et al., 2015; Chen et al., 2015; VoPham et al., 2015; Thrasher et al., 1993; Cosselman et al., 2015; Lebov et al., 2015; Piccoli et al., 2016; Garcia et al., 2017). Especially in young children pesticides exposure could harm the development of the central nervous and endocrine system. Two literature reviews supported by the European Food and Safety Authority (EFSA) showed significant associations between pesticide exposure and Parkinson’s disease and childhood leukaemia (EFSA, 2018; Ntzani et al., 2013). Moreover, a recent systematic review supported an association between residential exposure to pesticides and childhood brain tumors (Van Maele-Fabry et al., 2017). Urinary biological monitoring can be a suitable tool for exposure assessment; urine collection is non-invasive, combines exposure from all sources and routes, and many non-persistent chemicals or their metabolites are excreted into urine. Furthermore, compared to blood sampling, urine collection is generally accepted by parents and relatively easy to achieve in a non-clinical setting. However, urine collection from non-
toilet-trained children remains methodologically and practically challenging (Needham and Sexton, 2000; Bradman and Whyatt, 2005; Lee and Arbuckle, 2009). Collection methods should not introduce contamination or affect the integrity of the urine sample, should be convenient for young children and their parents, and should be cost-beneficial in large-scale biological monitoring studies. In addition, the minimum sample volume for analysis, the timing of collection, and the sample success rate should be considered (Lee and Arbuckle, 2009). Various methods have been widely used for urine collection in children, such as urine bags, the clean-catch, cotton diapers, gel diapers and absorbent pads (e.g. gauze or cotton pads). According to the National Institute for Health Care Excellence (NICE), the clean-catch is considered as the gold standard in a clinical setting (NICE, 2007). It is well known that the extraction of urine from a gel-absorbent polyacrylate diaper is complex. At the same time, these diapers are commonly used all over the world, and are therefore a potentially attractive collection device. Urine collection from an absorbent diaper insert or a urine bag has been used in the clinic for the diagnosis of urinary tract infections. Each method has its own advantages and limitations and it depends on the situation which method is preferred (Lee and Arbuckle, 2009; Liaw et al., 2000). The aim of this study was to examine and evaluate for non-toilet-trained children using different urine collection methods that could be applied in a non-clinical setting. Evaluation was quantified by user ratings on a 0–10 scale (0 = inconvenient, 10 = convenient) and the rate of successfully collected samples. Subsequently, the best method was validated for assessing tebuconazole pesticide exposure in the urine of young children, using hydroxy-tebuconazole as the major human urinary metabolite (Fustinoni et al., 2014; Mandic-Rajcevic et al., 2015).

2. Methods

2.1. Urine collection methods

Four commonly applied approaches to collect urine from non-toilet-trained children were studied for feasibility to collect urine samples from young children (age 0–3) in a non-clinical setting. The selected approaches were a urine collection pad (Hessels + Grob, Apeldoorn, the Netherlands), a urine bag (Urinocol Pediatric, Braun), the clean catch and a disposable polyacrylate diaper (Pampers Baby Dry size 3, Procter & Gamble). The advantages and limitations have been previously described by Lee and Arbuckle (Lee and Arbuckle, 2009)). In short, use of a collection pad as a diaper insert is relatively easy to apply, removal of the pad with absorbed urine is simple, and the urine extraction method is simple. Obtaining sufficient volume for urinalysis is a limitation of this method, especially when a relatively large volume (> 5 mL) is required for performing multiple analyses on one sample. We evaluated a relatively new type of collection pad, the PeeSpot, which has been validated for the diagnosis of urinary tract infections and electrolyte disturbances in a clinical environment. It consists of a felt material containing a dried hygroscopic polymer and can absorb up to 1.2 mL of urine (MAMA et al., 2013). The standard size is 0.5 × 2.4 × 1.0 cm (h × l × w). For this study the PeeSpot size was enlarged to 0.5 × 10.0 × 3.0 cm (h × l × w) with a capacity to absorb up to 15 mL. The urine bag has been commonly used in hospitals and is relatively easy to use, but needs more efforts from the parents compared to the PeeSpot. The bag should be attached to the skin in the correct position and after removal the urine should be transferred to a urine container. The parents have to monitor whether the adhesive tape detach or causes skin reactions (Lee and Arbuckle (2009)). The clean catch method is considered the gold standard for non-toilet-trained children. A void is collected by placing a container into the urine stream, e.g. during a regular diaper change or when bathing. This method might be time consuming, not always successful and it requires the involvement of preferably two adults, one to hold the child upright and one to catch the urine in the collection cup (Lee and Arbuckle (2009); NICE, 2007). The last collection method comprises the extraction of urine from a disposable polyacrylate or gel diaper. The Pampers baby dry diaper was selected as this type is available in many countries and consists of a separate gel compartment, which makes it easier to remove the material. Collection and storage of a wetted diaper in a sealed bag is easy and a normal routine for most parents or care takers. However, extraction of the urine from the diaper in the laboratory is more challenging (Hu et al., 2004).

2.2. Study design

A pilot study was conducted for end-user evaluation of the selected approaches. After ethics approval of the study protocol by the Medical Ethical Committee of the Radboud university medical centre, participants were recruited in the area of Nijmegen by placing posters. Written informed consent of each parent was obtained after verification of inclusion and exclusion criteria of the child. Parents and participants (n = 8, four boys and four girls) were asked to collect a urine sample on three consecutive days for each method. The sequence of the method application was randomly assigned. Written instructions and collection materials were provided. All urine collection materials were kept at 4 °C, and after all attempts were completed, the materials were stored at −20 °C in the laboratory. The urine bag and the PeeSpot were installed during a diaper refreshment to decrease participant burden. Parents were asked to check the diaper every 30 min after installation. According to the provided instructions, the parents had to place the PeeSpot in the area of the urethra. The clean catch method was performed during a regular diaper change. One pre-weighted diaper per day was transferred into a sealed bag and stored at room temperature. Parents were also asked to complete a short questionnaire regarding information on leakage, displacement, user experiences, and the overall convenience for the child and the parents, based on a user rating score on a 0–10 scale where 0 was inconvenient and 10 was convenient. A successful sample was defined as a yielded volume of at least 5 mL for urine analysis and free of feces contamination.

2.3. Urine extraction disposable diaper

Urine extraction from a diaper has been described by use of different techniques, e.g. using a syringe to aspirate the sample, immersing the gel layer in an organic solvent or by applying hydraulic pressure (Lee and Arbuckle, 2009). For the polyacrylate or gel diaper, the addition of calcium salts effectively collapses the polymer to release the urine. Hu et al. (2004) concluded that diapers containing a separate compartment with the gel material are most suitable for urine extraction, however they also concluded that the brand of the diaper could affect the recovery efficacy (Hu et al., 2004). In this study we adopted and modified the method of Hu et al. (2004), which was developed for the analysis of urinary pyrethroid metabolites. In short, the polyacrylate granules are coiled when dry and become uncoiled when the material comes in contact with water or urine. The polymer continues to uncoil, absorbs more water and forms a gel-like material. Upon addition of Ca2+ the polymer collapses due to ion exchange, and most of the absorbed urine is released with the analytes remaining in solution (Hu et al., 2004).

2.4. Analytical procedure

2.4.1. Chemicals and regents

Hydroxy-tebuconazole (TEB-OH; purity > 98%) and the internal standard (IS) D6-hydroxy-tebuconazole (D6-TEB-OH; purity > 98%) were obtained from Alsachim (Illkirch Graffenstaden, France). Calcium chloride (CaCl2, purity 99%) was obtained from Sigma-Aldrich (Zwijndrecht, the Netherlands). High performance liquid chromatography (HPLC) grade methanol (99.9%) was purchased from VWR chemicals (Fontenay-sous-Balis, France). Ultra HPLC grade acetonitrile
(99.9%) was obtained from Boom (Meppel, the Netherlands). Formic acid (98%) and acetic acid (100%) were obtained from Merck KGaA (Darmstadt, Germany). β-Glucuronidase/arylsulfatase aqueous solution from Helix pomatia was purchased from Roche Diagnostics (Mannheim, Germany). Purified water was obtained using a Millipore Milli-Q Advantage A10 system (Milford, MA, United States).

2.4.2. Preparation of standard solutions

Stock solutions of TEB-OH and D6-TEB-OH were prepared in methanol at concentrations of 2 mg/mL, and were diluted in methanol to working solutions of 1 μg/mL. All standards were stored at −20 °C in the dark. Additional working solutions of 100 ng/mL were freshly prepared in 95% water and 5% methanol (% v/v) and stored at 4 °C in the dark. The calibration curve was prepared by addition of an equal amount of standard solution to a mix of five urine voids collected from random persons. The calibration curve was treated equally as the sample extracts.

2.4.3. Experiments and preparations of extracts

A series of diapers was wetted with 50 mL of spiked urine on six levels in a concentration range of 0.05 to 25 ng/mL of TEB-OH, all in duplicate. Urine was extracted from the diaper according to the method of Hu et al. (2004), with slight modifications. Instead of a 150 g/L CaCl₂ solution, solid CaCl₂ was deposited directly onto the removed granules corresponding to 0.1 g CaCl₂ per gram of diaper material. In brief, after wetting a spot with 50 mL of spiked urine on the intact diaper, the polyacrylate containing part was opened. Twenty g of gel material was removed and transferred into a test tube. Two g of CaCl₂ was added and the tube was placed in a mechanical shaker for 30 min. The released urine (approx. 10–15 mL) was transferred to a clean tube, and was centrifuged for 5 min at 2000×g to remove remaining cotton fibers. Five mL of extracted urine was used to load the sample on a Waters Sep-Pak C18 solid phase extraction cartridge, and was eluted with 5 mL of undiluted methanol. After evaporation under a gentle flow of nitrogen (0.8 bar) the samples were reconstituted in 1 mL of methanol. All of the extraction procedures were performed on the same day.

2.4.4. Sample analysis

For the determination of TEB-OH, an aliquot of 2.5 μL was analyzed on a Waters Acquity H-Class liquid chromatograph (LC) (Waters, Milford, MA, USA) system equipped with a quaternary pump. The LC was coupled to a Waters TQ-S micro (Waters, Milford, MA, USA) system equipped with a quaternary pump. The LC on a Waters Acquity H-Class liquid chromatograph (LC) (Waters, Milford, MA, USA) was operated in multiple reaction monitoring (MRM) mode. Applied flow of nitrogen was 1.0 L/h, and a cone nitrogen flow of 50 L/h. The mass spectrometer was operated in multi reaction monitoring (MRM) mode. Applied mass transitions, collision energies and retention times for TEB-OH and IS are shown in Table 1. The mass transition selected for quantification TEB-OH was 325.02 → 69.96, and for qualification 325.02 → 124.97.

<table>
<thead>
<tr>
<th>Compound</th>
<th>RT (min)</th>
<th>Precursor ion (m/z)</th>
<th>Product ion (m/z)</th>
<th>Collision energy (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEB-OH</td>
<td>1.76</td>
<td>325.02</td>
<td>69.96</td>
<td>20</td>
</tr>
<tr>
<td>D6-TEB-OH</td>
<td>1.76</td>
<td>331.02</td>
<td>69.96</td>
<td>20</td>
</tr>
</tbody>
</table>

Creatinine was analyzed after urine extraction and centrifuging but prior to further pre-treatment of the samples by the laboratory for clinical chemistry of Radboud university medical centre according to the Jaffe method (Slot, 1965).

2.5. Method validation

Validation procedures were performed according to the guidance document on analytical quality control and method validation procedures for pesticide residues and analysis in food and feed (SANTE 11945/2015) (EU, 2017). Diaper recoveries and calibration curves were constructed by pouring an aliquot of urine spiked with TEB-OH and D6-TEB-OH onto the diaper and a comparison was made with the same aliquots that were not poured onto the diaper. In addition, creatinine was measured before and after the extraction procedure to assess whether creatinine correction of spot urine samples can be applied for the diaper method (Mage et al., 2008). The diaper matrix effect was evaluated by the difference in slope of the calibration curve with and without use of the diaper. Precision of the method was calculated as the relative standard deviation (RSD) of the extraction procedure at 5 ng/mL (performed in tenfold). Blank urine samples were added to the experiments to test the specificity of the method. The limit of quantification (LOQ) was defined as the lowest concentration of the analyte that can be measured with sufficient accuracy (RSD < 20%).

2.6. Assessment of the overall procedure

To assess the real world application of the overall method and the ability to detect tebuconazole metabolites in young children, seven different parents collected and provided a first morning diaper of their child (median age 5 months, min 3 months, max 29 months). The extraction procedure was performed as described and TEB-OH was determined with LC-MS. As these urine specimens were unspiked, the sulfate and glucuronide conjugates were deconjugated after diaper extraction with an excess of Helix Pomatia enzymes at 37 °C for 16 h. The concentration of the samples was estimated with a matrix-matched calibration curve which was treated equally as the urine samples.

2.7. Data analysis

Recovery and measurement results were presented as means ± standard deviation (SD) and calculations were performed with Microsoft Office Excel 2007. Linear calibration curves were created using a 1/x weighting factor, by plotting the area ratio of TEB-OH/D6-TEB-OH versus the concentrations of the standards.

3. Results

3.1. Urine collection methods

The median age of boys and girls in the pilot study was 20 months and 14 months, respectively. In total there were 24 attempts per collection method and the outcome measures of the pilot study are presented in Table 2. The yielded urine volume for each method was determined and was highest for the diaper (mean 120 mL). The PeeSpot yielded the least volume with an average of 4.1 mL. All of the samples collected with the clean catch method were free of feces; for the diaper it was 67%. The clean catch gave the highest percentage of missing samples (94%) and the diaper the lowest (42%). The diaper method showed the highest success rate with 67%, followed by the urine bag (21%), the PeeSpot (17%) and the clean catch (4%). Participant scores showed the highest success rate with 67%, followed by the urine bag (21%), the PeeSpot (17%) and the clean catch (4%). Participant scores were highest for the diaper and the PeeSpot with an 9.0 and 7.3, respectively. The major user comments for the PeeSpot were that the pad might be softer and therefore more comfortable for the child, that it was difficult to remove the pad from the diaper, and one parent reported skin irritation. The commentary on the urine bag method was that it
could detach from the skin resulting in leakage, and that the adhesive tape strip causes skin irritation when used repeatedly. Remarks regarding the clean catch illustrated that it requires at least two persons, one to hold the child and the other to catch the urine. Moreover, micturition of a young child on command remains a challenge. Participants commented that the risk of feces contamination is relatively high with the diaper method and one parent did not want to change the diaper brand for the study.

### 3.2. Method validation

The LOQ for TEB-OH in deconjugated urine was 0.1 ng/mL. Recoveries and precisions at five different levels are shown in Table 3. The percent recovery was on average 106% ± 3% for TEB-OH corrected with IS and with an RSD of 3%. The mean recovery of creatinine was 87% with an RSD of 3%. The mean creatinine level without the diaper was 5.2 ± 0.1 mmol/L, and with the diaper 4.5 ± 0.1 mmol/L. Precision of the procedure was within the limits (RSD < 20%) regarding the SANTE guidelines.

Blank extracts were all below the LOQ after the complete extraction procedure, indicating that the selected type of diaper is free from the analytes. The non-spiked deconjugated samples showed an average background level of 0.62 ng/mL of TEB-OH, indicating the level of TEB-OH in the urine mix of the calibration curve. The ratio of the slope of a calibration curve before diaper extraction compared to the slope after extraction in absence of an IS was considered as the diaper matrix effect. The matrix effect was estimated to be 40%, indicating a 40% lower TEB-OH response in the diaper extracts. Despite the decreased absolute response in the diaper samples, the response is adjusted by the IS (Table 3).

A Bland-Altman analysis of the uncorrected pre and the post diaper extraction revealed that the selected type of diaper is free from the analytes. When including the IS corrected values, the recovery of creatinine from the diaper was improved compared to previous study in a clinical setting of Alam et al. (2005). This simple and reliable test panel reported various outcomes with an RSD of 23% and we found a recovery of 87% (RSD 3%). The slope after diaper extraction compared to the slope after calibration curve before diaper extraction indicated that the selected type of diaper is free from the analytes. Using a validated diaper brand and type is essential to ensure high recoveries, but some parents may still not want to use the selected study diaper. The success rate of the clean catch method was in our study rather low compared to previous reports. A success rate of 88% has been reported by Alam et al. compared to 4% in our study. However, all of the children in the study of Alam et al. (2005) were admitted to the hospital, whereas in our study urine was collected at home. Nurses might be more successful in applying clean catch urine collection compared to the parents of the child. The success rate of the clean catch might be improved by stimulation of the suprapubic area with cold water, but this requires extensive training of the parents.

The success rate of PeeSpot was only 17%, while the device was found to be reliable in clinical applications (MAMA et al., 2013). This low success could be partly explained by the relatively low volume yield; 64% of the received samples yielded a volume below the required 5 mL. Despite the clear instructions, it might be that the position of the pad in the diaper was not optimal resulting in a lower volume absorbed, or that the pad was presumably displaced as a result of crawling/walking. The success rate of the PeeSpot might be increased by a training program for the parents and/or by accepting a sample volume lower than 5 mL. One parent reported that the PeeSpot caused skin irritation and for that reason discontinued to use this method. The amount of feces contamination was low compared to the diaper method because the diaper with PeeSpot was frequently checked as advised for collection pads (Weuve et al., 2006; Dorey and Zimmermann, 2008).

Urine bags are frequently used in hospitals and are often applied in clinical studies. The risk of contamination is low, which makes it a suitable method for the determination of urinary tract infections (NICE, 2007). Although it is reported that the use of urine bags is relatively simple and reliable, the test panel reported various outcomes with an overall high number of missing samples (67%), compared to only 4% in a previous study in a clinical setting of Alam et al. (2005). Figure S1 shows how the procedure was performed, with the equipment attached to the diaper. The recovery of creatinine from the diaper was improved compared to the method applied by Hu et al. (2004). They reported a recovery of 71% (RSD 23%) and we found a recovery of 87% (RSD 3%). The improvement might be accounted by the use of solid CaCl2 rather than a solution, which likely dilutes the urine aliquot. However, the diapers employed by Hu et al. were from a different manufacturer compared to the diaper method employed by Alam et al. (2005).

### 3.3. Assessment of the total procedure

Seven diapers were analyzed for TEB-OH to assess the overall procedure, including sample collection and extraction. Three of the seven samples were above LOQ and the mean TEB-OH concentration in these samples was 0.72 ± 0.36 ng/mL. Although it was a small sample population, we noticed that the three positive urine samples were from children who consumed solid food. Urine samples with levels below the detection limit were from children with a diet of only breast milk or bottled milk. With this method, tebuconazole exposure can be quantified in young children.

### 4. Discussion

This study evaluated four approaches to collect urine from non-toilet-trained children for pesticide exposure characterization. The method should be suitable for urine collection at home, and the collection material must be acceptable and the urine collection itself should impose a low burden on both parents and child. A minimum sample volume of 5 mL is required for multiple laboratory analysis. The diaper method demonstrated to be most successful and the participant scores were highest of all four approaches tested. Collection of urine with the diaper succeeded in 67% of the total number of attempts, and feces in the diaper caused all missed samples. Participant scores were highest of all four approaches tested. Collection of urine with the diaper succeeded in 67% of the total number of attempts, and feces in the diaper caused all missed samples. Participants were instructed to store only one diaper per day. For biomonitoring purposes it is advised to instruct the participants to collect the next diaper if the first attempt is not successful. This method was in our study rather low compared to previous reports. A success rate of 88% has been reported by Alam et al. compared to 4% in our study. However, all of the children in the study of Alam et al. (2005) were admitted to the hospital, whereas in our study urine was collected at home. Nurses might be more successful in applying clean catch urine collection compared to the parents of the child. The success rate of the clean catch might be improved by stimulation of the suprapubic area with cold water, but this requires extensive training of the parents.

The success rate of PeeSpot was only 17%, while the device was found to be reliable in clinical applications (MAMA et al., 2013). This low success could be partly explained by the relatively low volume yield; 64% of the received samples yielded a volume below the required 5 mL. Despite the clear instructions, it might be that the position of the pad in the diaper was not optimal resulting in a lower volume absorbed, or that the pad was presumably displaced as a result of crawling/walking. The success rate of the PeeSpot might be increased by a training program for the parents and/or by accepting a sample volume lower than 5 mL. One parent reported that the PeeSpot caused skin irritation and for that reason discontinued to use this method. The amount of feces contamination was low compared to the diaper method because the diaper with PeeSpot was frequently checked as advised for collection pads (Weuve et al., 2006; Dorey and Zimmermann, 2008).

Urine bags are frequently used in hospitals and are often applied in clinical studies. The risk of contamination is low, which makes it a suitable method for the determination of urinary tract infections (NICE, 2007). Although it is reported that the use of urine bags is relatively simple and reliable, the test panel reported various outcomes with an overall high number of missing samples (67%), compared to only 4% in a previous study in a clinical setting of Alam et al. (2005). Figure S1 shows how the procedure was performed, with the equipment attached to the diaper. The recovery of creatinine from the diaper was improved compared to the method applied by Hu et al. (2004). They reported a recovery of 71% (RSD 23%) and we found a recovery of 87% (RSD 3%). The improvement might be accounted by the use of solid CaCl2 rather than a solution, which likely dilutes the urine aliquot. However, the diapers employed by Hu et al. were from a different manufacturer compared to the diaper method employed by Alam et al. (2005).
Oya et al. (2017) reported recoveries for organophosphate metabolites from a diaper ranging from 54% to 101% for dimethyl dithiophosphate and diethyl phosphate, respectively (Oya et al., 2017). The recoveries of pyrethroid metabolites reported by Hu et al. (2004) were between 44% and 122%, and recovery data from Saito et al. (2014) were between 10% and 123%. The recoveries for TEB-OH in this study were 26% without an IS and 106% with an IS. The reproducibility of the method was good, precision levels were higher or comparable to previous methods and within the limits of the guidelines (Hu et al., 2004; EU, 2017, 2010; Saito et al., 2014; Bakker et al., 1999). The large difference in recoveries could be explained by the high amounts of CaCl2 in the aliquots. This may have affected the SPE efficacy or even had led to ESI suppression. A Bland-Altman analysis revealed an absolute mean of differences of almost zero, indicating that the TEB-OH measurement is reliable after diaper extraction and the addition of an IS. The use of an internal standard is therefore strongly recommended in this type of extractions.

The method described in this paper focused on the collection of spot urine samples in non-toilet-trained children. Based on the experiences in this study, collection of 24-hour urine seems very challenging, as it will result in many lost samples and in the case of diapers it will result in considerable number of diapers being contaminated with feces. For most biomonitoring studies, spot urine samples are manageable and affordable, and correction for urine dilution with creatinine is common practice (Mage et al., 2008; Fenske et al., 2000). For all analytes it should be verified whether the diaper material is free from the contaminants of the compounds of interest (Goodpaster et al., 2011). The blank samples showed that the Pampers Baby Dry diaper is free from TEB-OH.

Additionally, we showed in a small test panel that exposure to tebuconazole can be assessed by applying the diaper method. The average TEB-OH level was 0.72 ng/mL. Although it was a small sample population, it is noteworthy for further research that the urine samples were evaluated for biomonitoring purposes of tebuconazole in non-clinical setting. Use of a commercially available disposable diaper was reliable after diaper extraction and the addition of an IS. The use of an internal standard is therefore strongly recommended in this type of extractions.

In this study, four methods to collect urine from non-toilet-trained children were evaluated for biomonitoring purposes of tebuconazole in a non-clinical setting. Use of a commercially available disposable diaper was the most suitable and convenient method for the parents and the child. A previously described diaper urine extraction procedure was improved and the rapid extraction method results in high recoveries of TEB-OH and creatinine. This method can serve as a basis for extension to other substances of interest.

Conflict of interest

The authors report no potential conflicts of interest.

Funding

This work was supported by the Dutch National Institute for Public Health and the Environment (RIVM).

Acknowledgement

We would like to thank all participants and their parents who tested the urine collection approaches or provided a diaper.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxlet.2018.09.018.

References

Nizami, E.E.C.M., Nitrinos, G., Evangelou, E., Tsoulaki, I., 2013. Literature review on epidemiological studies linking exposure to pesticides and health effects. EFSA.
Saito, S., Ueyama, J., Kondo, T., Saito, I., Shiibata, E., Gotoh, M., et al., 2014. A non-


