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High-throughput and sensitive determination of urinary zearalenone and metabolites by UPLC-MS/MS and its application to a human exposure study

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Abstract

Biomarker-based strategies to assess human exposure to mycotoxins have gained increased acceptance in recent years. In this study, an improved UPLC-MS/MS method following 96-well μElution solid-phase extraction was developed and validated for the sensitive and high-throughput determination of zearalenone (ZEN) and its five metabolites, α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), α-zearalanol (α-ZAL), β-zearalanol (β-ZAL) and zearalanone (ZAN) in human urine samples, using ^{13}C-ZEN as internal standard for accurate quantification. Two plates of samples (n=192) could be processed within 2 h; and baseline separation of all the analytes were achieved with a total run time of 6 min. The proposed method allowed ZEN and its metabolites to be sensitively determined in a high-throughput way for the first time, which significantly improved efficiency and accuracy with respect to existing methods. The limits of detection (LODs) and limits of quantitation (LOQs) ranged from 0.02 to 0.06 ng mL^{-1} and from 0.05 to 0.2 ng mL^{-1}, respectively. The recoveries for the spiked samples were from 87.9% to 100%, with relative standard deviations (RSDs) less than 7%. 301 urine samples collected from healthy volunteers aged

0-84 years in China were analyzed both with and without enzyme hydrolysis to determine total and free ZEN biomarkers, respectively. ZEN, ZAN, α-ZEL and β-ZEL were detected in 71.4% of the samples ranged 0.02-3.7 ng mL^{-1} after enzyme hydrolysis. The estimated mean probable daily intake (PDI) was largely below the tolerable daily intake (TDI). Adolescents had higher exposure than children, adults and elderly.

Keywords

Biomonitoring, Zearalenone, Metabolites, Urine, 96-well μElution SPE, UPLC-MS/MS

Introduction

Zearalenone (ZEN) is a naturally existing estrogenic mycotoxin produced mainly by *Fusaruim graminearum*, *Fusarium culmorum, Fusarium equiseti* and *Fusarium sacchari* [1,2]. It commonly occurs in various cereal crops and processed grains, and can also be found in animal-derived food as a consequence of a carry-over from contaminated feeds. ZEN, while being of low acute toxicity, gives rise to major concerns about distinct estrogenic effects, resulting in adverse impacts on genital organs and reproductive system of mammalian species [2-5]. In this regard, JECFA set the provisional maximum tolerable daily intake (PMTDI) for ZEN at 0.5 μg/kg bw/day [6], while EFSA making the tolerable daily intake (TDI) of 0.25 μg/kg bw/day [7]. Many organizations and countries have established maximum limits for ZEN in food ranging from 30 to 1000 μg/Kg [8,9], based on these health-based guidance values (HBGVs) with reference to their own food consumption databases.

After oral administration, ZEN is rapidly absorbed and subsequently degraded primarily into α-zearalenol (α-ZEL) and β-zearalenol (β-ZEL) [1,10,11], which undergo a further reduction to α-zearalanol (α-ZAL) and β-zearalanol (β-ZAL) [12,13]. The resulted α-ZAL is found to be metabolized into its isomer β-ZAL and, to a lesser extent, into zearalanone (ZAN) [14]. These metabolites are partially conjugated with sulfonic or glucuronic acid and excreted in the urine [3].

Humans are easily exposed to ZEN through the diet [15]. Evaluation of the exposure levels has been traditionally performed based on occurrence data combined with consumption data [16-19].

More recently, considering the heterogeneous distribution of mycotoxins in foodstuffs [20] and a potential underrepresentation of the used consumption databases [21], biomarker-based approaches have been proposed and gained increased acceptance. Directly monitoring the presence of ZEN and its metabolites in human physiological samples provides an advanced tool to obtain the actual exposure of an individual or a subgroup of population, thereby facilitating an improved comprehensive assessment [22]. In the circumstance, the development of analytical methods in response to the current needs is strongly recommended.

Various methods for the analysis of ZEN and ZEN metabolites have been established for biological samples, based on high-performance liquid chromatography (HPLC) [23-26], gas chromatography-mass spectrometry (GC-MS) [27], liquid chromatography-mass spectrometry (LC-MS) [28] and liquid chromatography-tandem mass spectrometry (LC-MS/MS) [29-33]. Among these methods, LC-MS/MS increasingly became the preferred technique due to its high sensitivity and selectivity, and was satisfactorily implemented to test human urine samples. Nevertheless, some of the applications limited their determination to only ZEN [34] or its major metabolites (α-ZEL and β-ZEL) [35-37]. In addition, since ZEN and its metabolites were easily conjugated with glucuronic acid *in vivo*, some following studies made further consideration of the resultant conjugations, i.e. ZEN-14-glucuronic acid (ZEN-14-GlcA), ZAN-14-GlcA, and α/β–ZEL-14-GlcA, which were included in their LC-MS/MS methods [38,39]. However, the relatively low sensitivity to the conjugations (LOQ: 1~25 ng/mL) greatly challenged the practical usage of these methods in detection of urinary biomarkers at environmentally relevant concentration levels. As a consequence, an alternative strategy has been successfully developed by measuring the total (free + conjugated) amount of each analyte after enzymatic deconjugation. The most relevant works were reported recently to determine ZEN and its five metabolites in human urine, involving enzymatic hydrolysis, sample extraction/cleanup, and UPLC-MS/MS quantification [30,33]. These methods achieved a high increase in sensitivity, but the drawbacks of labor-intensive and time-consuming preparation procedures (e.g. liquid–liquid extractions, use of solid-phase extraction (SPE) columns, evaporation and reconstitution steps) limited their further application in large-scale sample analysis. To address such an issue, a 96-well μElution plate was introduced in this work, for the first time, allowing for

the simultaneous preparation of multiple samples and meanwhile reducing the amount of solvent consumed.

This paper presented a sensitive and high-throughput method for the determination of ZEN and its five metabolites (total or free) in human urine samples by UPLC-MS/MS combined with a PRiME HLB 96-well μElution plate that enabled the simultaneous multi-sample processing. Within a total run time of 6 min, ZEN and its metabolites were baseline-separated, highly enhancing the selectivity of the method. After being validated according to the guidelines defined by the EMEA [40] and FDA [41], the developed method was implemented in analysis of 301 human urine samples collected from healthy individuals in China. The advantages of high-throughput, sensitivity, and accuracy have made the proposed method a powerful tool for large-scale analysis to support ZEN-related toxicokinetic studies, bio-surveillance and exposure risk assessment.

Methods

Chemicals and materials

Certificated standard solutions of ZEN (100 μg/mL), α-ZEL (10 μg/mL), β-ZEL (10 μg/mL), ZAN (10 μg/mL), α-ZAL (10 μg/mL), β-ZAL (10 μg/mL) and $^{13}C_{18}$-ZEN (3 μg/mL) were purchased from Biopure (Tulln, Austria) and stored at -40 ºC in the dark. β-glucuronidase (from E coli.) was from Sigma-Aldrich (MO, USA). Acetonitrile, methanol, ammonia acetate, formic acid and acetic acid were of LC/MS grade (Fisher Scientific, Leicestershire, United Kingdom). All other chemicals were of analytical grade or better. The deionized water (18.2 MΩ cm) was collected from a Milli-Q system (Millipore Corp., Bedford, MA). The Oasis PRiME HLB 96-well μElution plate (3 mg of sorbent in each well) were obtained from Waters (Milford, MA, USA). A mixed standard solution containing 1 μg/mL of each analyte was prepared in acetonitrile and stored at 4 ◦C in the dark, remaining stable for at least six months. The working dilutions of mixed standards were prepared at each day of measurement. The enzyme solution was prepared by dissolving 14.4 mg β-glucuronidase (6.9×10^5 U g solid^{-1}) in 10 mL of 0.075 mol L^{-1} phosphate buffer (potassium phosphate dibasic + potassium phosphate monobasic, pH 6.8) freshly on the day of use.

Sample collection and storage

Morning urine samples were collected from healthy volunteers aged 0-84 years on three consecutive days in 2016 in Henan province, China (n=301; 107 males, 194 females), and were stored frozen at -70 ◦C. The urine from three days were mixed at a 1:1:1 ratio to make one sample prior to analyses. This study protocol was approved by the ethics committee of China National Center for Food Safety Risk Assessment (No. 2016030063); and all the methods were performed in accordance with the approved guidelines and regulations. The study was fully explained; and informed consents from the adult participants or parents on behalf of their children who participated in the study were obtained.

Preparation of calibration standards and quality control samples

The calibration standard solutions were prepared by serial dilutions of the mixed standard solution (1μg/mL of each analyte) with methanol/water (50/50, v/v) to final concentrations of 0.1, 0.2, 0.5, 1, 2, 5, 10 and 20 ng/mL, each solution containing 3 ng/mL ^{13}C-ZEN. Quality control (QC) samples at 3 concentrations (0.5, 2 and 20 ng/mL) were prepared by dilution of the mixed standard solution with blank urine samples, and stored at -70℃ until use. The QC samples were analyzed in each batch of the study samples, and their measured values should be within ±15% of the nominal values.

Sample preparation

Determination of free analytes. Urine samples were thawed completely and centrifuged at 5000×g for 15 min at 4 ºC. To 1 mL of the supernatant ^{13}C-ZEN internal standard was added at a concentration of 3 ng/mL, followed by a dilution with 1.5 mL of phosphate buffer (0.075 mol L^{-1}, pH 6.8). 500 μL aliquot of the diluted sample was loaded onto an Oasis ® PRiME HLB μElution Plate which was pre-conditioned with 200 μL methanol followed by 200 μL of water. The loaded samples were allowed to slowly pass through the sorbent under vacuum. The wells were sequentially washed with 200 μL of water and then 200 μL of 50% methanol to remove

interfering compounds. After drying the plate, the analytes were eluted twice with 50 μL each of methanol into a 96-well collection plate and then diluted with 100 μL water prior to LC-MS/MS analysis.

Determination of total analytes. After thawed, centrifuged and spiked with ^{13}C-ZEN internal standard, 1 mL of the urine sample was digested with 1000 Units of β-glucuronidase (dissolved in 1.5 mL phosphate buffer, 0.075 mol L^{-1}, pH 6.8) in a shaking water-bath at 37ºC for 18 h. Afterward, the digested samples were centrifuged again (5000×g; 15 min; 4ºC); 500 μL of the supernatant was loaded onto Oasis ® PRiME HLB μElution Plate and then treated by exactly the same procedure as described above.

LC-MS/MS analysis

Analysis was carried out on an ACQUITY UPLC™ I-Class system (Waters, MA, USA) coupled to a Xevo® TQ-S tandem quadrupole mass spectrometer (Waters, MA, USA). The instrument operation and data processing was performed on Masslynx software (version 4.1).

Chromatographic condition

Chromatographic separation of ZEN and its five metabolites was achieved on CORTECS™ UPLC® C18 Column (2.1×100 mm, 1.6 μm) from Waters (MA, USA). A gradient mobile phase consisting of water (solvent A) and methanol/acetonitrile (80/20, v/v, solvent B) was applied at a flow rate of 0.4 mL/min. The gradient program started with 50% B, which was 50%-66% at 0-4 min, then increased to 90% within 0.1 min, held at 90% for 1.9 min, and then reduced to 50% within 0.1 min and held for 1.9 min, with the total runtime of 6 min. The column temperature was kept at 40 °C; the autosampler temperature was 4 °C; and the injection volume was 10 μL.

Mass spectrometry condition

A Xevo® TQ-S tandem quadrupole mass spectrometer, equipped with ESI source, was used for mass detection and analysis. The MS/MS parameters in multi reaction monitoring (MRM) mode were optimized for each analyte by continuously infusing standard solutions into the mass spectrometer. The MRM transitions together with their corresponding optimum cone voltages and collision energies were presented in Table 1. Other optimized MS/MS parameters were: source temperature, 150 °C; capillary voltage, -2.80 kV; desolvation gas, nitrogen, 900 L h^{-1}, 500 °C; cone gas, nitrogen, 150 L h^{-1}; collision gas, argon, 0.15 mL/min.

Method validation

The method was validated in accordance with the guidelines defined by the EMEA [40] and FDA [41]. Linearity, selectivity, accuracy (method recovery, R_M), precision (intra and inter-day variability), sensitivity (LOD and LOQ) and carry-over were evaluated for ZEN and its five metabolites. R_M was investigated at low (0.5 ng/mL), medium (1 ng/mL) and high (5 ng/mL) spiking level in blank urine with $^{13}C_{18}$-ZEN internal standard correction. The evaluation of apparent recovery (R_A), extraction recovery (R_E), and matrix effects (signal suppression/enhancement, SSE) was performed using three types of calibration curves as follows [42]: calibration curve prepared in initial mobile phase (I), matrix-matched calibration curves prepared by spiking before (II) and after sample preparation (III). The R_E and R_A were calculated by dividing the slope of calibration curve II by the slopes of calibration curve III and calibration curve I, respectively. The SSE was determined by comparing the slope of calibration curve III with that of calibration curve I.

Statistical analysis

For statistical tests, undetectable ZEN biomarker concentration was set as half the value of their respective LOD. The concentration values of total ZEN were natural log transformed for normality and then analyzed with independent sample t-test and ANOVA to determine the differences among different subgroups (age, gender). Statistical analysis was performed using SPSS, version 19 (SPSS, Chicago, IL, USA). A p-value < 0.05 was considered as statistically significant.

Results and Discussion

Optimization of MS/MS conditions

Optimization of the MS/MS conditions was performed by direct infusions of each individual compound. Ionization mode, capillary voltage, cone voltage, cone gas flow, source temperature, desolvation gas flow and desolvation temperature were manually optimized in steps to achieve the most intense response of the precursor ion. ESI in negative mode with the capillary voltage of -2.8 kV was selected; and $[M-H]^-$ was chosen as the precursor ion for all analytes of interest. Afterwards, the collision energy (CE) was tuned to produce the most sensitive and stable product ion in the collision cell. For each compound of interest two MRM transitions were selected and optimized, one for quantification and another for identification, as listed in Table 1.

Chromatographic separation

The closely similar structures of ZEN and its metabolites challenged their chromatographic separation. The baseline-separation of the six target compounds have not been achieved in previous works [30,31,33,43,44]. To obtain a satisfactory UPLC separation, the main variables affecting UPLC behavior were studied, including UPLC column, organic mobile phase (acetonitrile, methanol, or mixture of acetonitrile and methanol), additives (ammonium acetate, ammonium formate, acetic acid and formic acid) at different concentrations, and other parameters, such as the flow rate and gradient program. Among the tested columns, CORTECS UPLC C18 column (2.1 mm×100 mm, 1.6 μm) from Waters (Mildford, MA, USA) provided the best resolution and peak shapes for all the target compounds, and was consequently selected for further study. The organic modifier in the mobile phase markedly affected the chromatographic separation. The baseline-separation of ZAN and α-ZEL was hardly achieved with methanol alone as organic modifier. And it also happened to the separation of ZEN and ZAN, when acetonitrile alone was used. Accordingly, the mixture of methanol and acetonitrile

(80/20, v/v) was optimized as the organic solvent, providing a complete separation and sharp peaks of all the analytes in a 6-min gradient elution. Formic acid, ammonium formate, acetic acid and ammonium acetate were evaluated as additives, giving no contribution to the chromatographic separation and a very slight influence on ion response. A representative chromatogram of a standard mixture of the target compounds at 0.2 ng mL^{-1} was illustrated in Fig. 1.

Sample preparation

SPE as a powerful technique for sample preparation has been widely used in ZEN analysis. However, the requirement of laborious evaporation and reconstitution steps greatly limited its utility in fast analysis. To circumvent these obstacles, a 96-well PRiME HLB μElution plate was introduced for the first time to extract ZEN and its five metabolites from human urine samples. The main parameters including loading, washing, and elution buffer were optimized to improve the efficiency, selectivity, and sensitivity.

Urine matrix containing multiple endogenous components and metabolites, may cause complex background signals or increase the risk of clogging. Accordingly, pre-dilution of sample was necessary to achieve a better retention of target compound on the PRiME HLB μElution plate. After optimization, urine samples were 1.5-fold diluted in phosphate buffer (0.075 mol L^{-1}, pH 6.8), which is also the preferred solvent of β-glucuronidase as recommended by the manufacturer, and then loaded onto the μElution plate, resulting in a complete retention of the analytes.

The selection of washing and elution buffer was a crucial step to reduce the matrix effect and increase the recovery. Spiked urine samples (containing 10 ng mL^{-1} of each analyte) both before and after enzymatic hydrolysis were used for the optimization studies. After sample loading, a wash with pure water was necessary to remove salts and other water-soluble impurities. After that, buffers consisting of varying levels of methanol (5, 10, 20, 30, 40, 50, 75, 90 and 100%, v/v) were applied to rinse the μElution plate, with effluent being collected and analyzed for target compounds. As evidenced in Fig. 2, all the analytes started to be washed off with 50%

methanol, and were completely eluted with 100% methanol. Therefore, 50% methanol and 100% methanol were adopted as the washing and elution buffer respectively, allowing the maximum removal of interferences while stably retaining the analytes of interest. The effect of elution volume was also evaluated with volumes from 25 to 200 μL (25, 50, 75, 100, 150 and 200 μL) in six replicates. High recoveries in the range of 94%~100% with RSD lower than 6.0% were obtained for all analytes with the use of 100 μL elution buffer. And no significant enhancement was observed with further increasing of elution volume. Eventually, the best performance was obtained by washing with 200 μL of water and then 200 μL of 50% methanol, followed by eluting twice with 50 μL each of methanol, achieving optimal extraction recoveries of 94%~116% and matrix effects ranging from 76.8~85.2%, for all the analytes.

The proposed 96-well μElution SPE protocol has several advantages over other approaches reported previously. The μElution plate containing only 2 mg sorbent in each well, drastically reduces the consumption of sample and reagent as well as the contamination from packing materials. Additionally, unlike "dilution and shoot" method [38,39], this procedure does not result in sample dilution, which might cause an apparent loss of sensitivity. To our knowledge, this is the first report that enables high-throughput sample cleanup for the determination of ZEN and its metabolites, allowing each plate of urine samples (n=96) to be processed within 1 h.

Method validation

The method was validated with reference to the guidelines specified by the EMEA and FDA, in terms of linearity, selectivity, sensitivity (LOD and LOQ), accuracy, precision (intra and inter-day variability) and carryover.

The linearity was determined in the range from the LOQ up to 20.0 ng mL^{-1} by analyzing calibration standards at eight concentration levels on three different days. Regression coefficients (R^2) of the calibration curves were ranged from 0.9984 to 0.9999, with deviations less than 12% for all measured concentrations. Standardized residuals from linear regression were also analyzed (see Supplementary Information, Figure S1), showing a random pattern. These results indicated good linear fits for all analytes.

Selectivity of the method was evaluated by comparing the chromatograms of 6 different blank urine samples with samples fortified with a mixture of analytes near the LOQ levels. As displayed in Fig. 3, no endogenous interferences were observed at the retention time of each analyte or internal standard. In addition, the baseline separation of the 6 target compounds further enhanced the selectivity of the method.

The LOD and LOQ of the method were determined using spiked blank samples at low levels, corresponding with the signal to noise ratio (S/N) greater than 3 and 10, respectively. The LOD and LOQ values for all analytes ranged 0.02-0.06 ng mL^{-1} and 0.05-0.2 ng mL^{-1}, with the relative standard deviations (RSDs) at LOQ levels of less than 20% (n=6) for all the analytes, as summarized in Table 2, representing a significant increase in sensitivity compared with the previously reported works [28,31,34].

Values of accuracy and precision were obtained from QC samples at three levels (0.5, 1.0 and 5.0 ng mL^{-1}) analyzed on three different days in six replicates. The accuracy, expressed as the method recoveries (R_M, quantification with IS) of known amounts of target compounds in QC samples, ranged between 87.9% and 100% for all concentration levels. And the intra-day and inter-day precisions (as RSD) were 1.2%-6.9% and 2.7%-10.7%, respectively (Table 3).

Additionally, the extraction recovery (R_E) and matrix effects (signal suppression/enhancement, SSE) were also investigated as mentioned above. The good R_E ranging from 94.1% for ZEN to 116% for β-ZAL and good SSE between 76.8% and 85.2% for all the analytes were obtained. It is worth mentioning that even without IS compensation, the recoveries (apparent recoveries, R_A) were still satisfactory and ranged between 78.0% and 93.5% (Table 2).

No sample-to-sample carryover was found upon sequential injections of high-concentration urine sample, followed by three consecutive blanks.

Since ZEN-related urinary biomarkers are in the low ng mL^{-1} range, sensitivity plays a critical role in ZEN exposure study. Up to now, only a few data are available. A pilot study involving 27 urine samples from Spain did not confirm the presence of ZEN at an LOD of 3 ng/mL [33]. A recent study of Gerding et al. detected α-ZEL in 2.8% of 142 samples from Haiti, whereas

306 no ZEN biomarkers were detected in 50 samples from Germany and in 95 samples from
307 Bangladesh [39]. The low frequency of positivity for ZEN and its metabolites, as stated by the
308 authors, might be attributed to the high LOD of their approach. In our study, due to the high
309 sensitivity of the method, high detection rate of 71.4% for the monitored ZEN biomarkers
310 ranged 0.02-3.7 ng mL^{-1} was achieved, which guarantee a reliable exposure study and risk
311 assessment.
312
313 ***Human biomonitoring***
314 Both free and total amounts of ZEN, α- ZEL, β-ZEL, ZAN, α-ZAL and β-ZAL in 301 human
315 urine samples collected in Henan province, China, was monitored using the developed method.
316 ZEN, ZAN, α-ZEL and β-ZEL could be detected, whereas α-ZAL and β-ZAL were not found
317 in any of the analyzed samples. Chromatograms of the detected analytes in a naturally
318 contaminated human urine were exemplarily shown in Fig. 4.
319 Without β-glucuronidase hydrolysis, only 3.3% (n=10/301) samples were positive, with the
320 detection rates of ZEN, ZAN, α-ZEL and β-ZEL being 1.3% (n=4/301), 0.3% (n=1/301), 1.3%
321 (n=4/301) and 1.0% (n=3/301). ZEN was quantified in only one sample at a very low level of
322 0.05 ng mL^{-1}. And other detected compounds were all below their respective LOQ.
323 For the total amounts, much higher detection rates of ZEN (71.1%), ZAN (1.0%), α-ZEL
324 (4.0%) and β-ZEL (21.9%) were obtained, with mean concentrations of 0.24 ng mL^{-1}, 0.017 ng
325 mL^{-1}, 0.035 ng mL^{-1} and 0.082 ng mL^{-1}, respectively (Table 4). In total, 28.6% of the samples
326 were negative for all monitored ZEN biomarkers.
327 To further clarify the distribution of ZEN and its metabolites within the population of this
328 study, the concentrations of these compounds in urine were analyzed by gender and 4 age
329 groups (0-12, 13-18, 19-65 and >65), as presented in Table 5. The mean level of tZEN was
330 slightly higher in female (0.27±0.39 ng mL^{-1}) than in male (0.19±0.25 ng mL^{-1}), but the difference
331 did not reach statistical significance (P=0.068). All the 4 age groups were positive for ZEN and
332 β-ZEL, while α-ZEL and ZAN were not observed in the elderly group. The mean level of tZEN
333 was highest in the adolescent group (age 13-18, 0.45±0.44 ng mL^{-1}, $P<0.05$). The other three groups

had significantly low amounts of tZEN, with the ranking as follows: children (age≤12, 0.27±0.49 ng mL^{-1}), adults (age 19-65, 0.21±0.27 ng mL^{-1}), then elderly group (age >65, 0.14±0.18 ng mL^{-1}). But no significant difference was observed (P=0.361, 0.221 and 0.066) among these three groups.

The analyte concentrations at ng mL^{-1} levels in human urine found in this study were in good agreement with those reported previously as summarized in Table 6. Direct approaches designed to monitor ZEN, its metabolites (e.g., α-ZEL and β-ZEL) and glucuronide conjugates (e.g., ZEN-14-GlcA, α-ZEL-14-GlcA, β-ZEL-14-GlcA) [36, 38, 39, 45-50] are generally less sensitive than indirect approaches determining ZEN and its metabolites after enzymatic treatment [32, 34, 51-54]. Direct methods without hydrolysis and enrichment can detect only high concentrations (positive rates < 8%), which may not be suitable for ZEN assessment. Using indirect approaches, biomonitoring of ZEN in Europe (Sweden [52], Germany [53] and Southern Italy [51]) indicates a very low ZEN exposure, with the mean levels of total ZEN ranging from 0.05 to 0.23 ng/mL. Although relatively higher mean values of total ZEN were recorded in the US [32] as well as some regions from South Africa [54]. The excretion patterns of ZEN were highly differentiated among countries. Samples from Tunisian women had detectable ZEN, α-ZAL and β-ZAL [34]; samples collected in South Africa and southern Italy were positive for ZEN, α-ZEL and β-ZEL [51,54]; and samples from New Jersey girls were found positive for ZEN and its five metabolites (α-ZOL, β-ZEL, α-ZAL, β-ZAL and ZAN) [32].

Probable daily intake of ZEN

Based on these findings, a probable daily intake (PDI) for ZEN could be calculated from the urinary concentrations of ZEN-related biomarkers based on published urinary excretion rates, using the following formula:

$$\mathrm{PDI} = \frac{C \times V \times 100}{W \times E}$$

where C = biomarker concentration (μg L^{-1}), V = daily urine excretion (L), W = body weight (kg), E = excretion rate (%).

ZEN metabolite concentrations were adjusted to equivalent ZEN concentrations. A mean body weight of 60 kg and a mean daily urine excretion of 1.5 L were assumed [55]. Since no data on human excretion for ZEN was available, the 24 h excretion rate measured in piglets (36.8%) was used to estimate the PDI in human [51,56]. The mean PDI for ZEN was determined to be 0.024 μg/kg bw, equivalent to approximately 5.0% of the PMTDI set by JECFA (0.5 μg/kg bw/day) [6] or 10% of the TDI set by EFSA (0.25 μg/kg bw/day) [7]. In another study, a urinary excretion rate of ZEN was determined to be 9.4% (free ZEN and ZEN-GlcA combined as total ZEN) from a 27 year old, healthy male volunteer [57]. Accordingly, the mean PDI for ZEN was deduced to be 0.061 μg/kg bw, around 12% of the PMTDI set by JECFA [6] or 24% of the TDI set by EFSA [7]. Both the estimations indicated a low health risk from ZEN exposure in the Chinese subpopulation. It is noteworthy that since the excretion rate of ZEN obtained from large-scale human studies was unavailable, the calculated PDI in this study were based on piglet excretion data or human excretion rate from a single individual, which consequently should be considered a rough estimate rather than an accurate risk assessment.

Conclusion

A rapid, sensitive and selective 96-well μElution SPE followed by UPLC-MS/MS method has been developed and validated for the determination of ZEN and its metabolites in urine samples. The application of a PRiME HLB 96-well μElution plate permitted rapid and simultaneous preparation of multiple samples without the need for evaporation and reconstitution steps. After detailed validation, the proposed method was implemented to determine the target compounds in human urine samples collected from healthy volunteers in China. ZEN, ZAN, α-ZEL and β-ZEL were detected both with and without β-glucuronidase hydrolysis. And due to the improved sensitivity, high detection rate of 71.4% was obtained for ZEN related biomarkers after enzyme hydrolysis. The mean PDI for ZEN was estimated to be 0.025 μg/kg bw, twenty times lower than the PMTDI set by JECFA, indicating a low health risk. Age-sex analysis of the participants implicated that the adolescent group had the highest exposure to ZEN. This well-

388 tuned method delivered significantly improved throughput, sensitivity, and specificity as well
389 as reductions in time consumption, sample usage, and waste generated, providing a powerful
390 alternative for large scale bio-surveillance and help in ZEN exposure risk assessment.
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547 Additional Information

548 The authors declare to have no conflict of interests.
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Figure Legends

Fig. 1 Extracted ion chromatograms of UPLC separation of the 6 analytes (0.2 ng/mL of each compound).

Fig. 2 Elution of ZEN and its metabolites from spiked urine samples before (a) and after (b) enzymatic hydrolysis with 5%~100% methanol.

Fig. 3 LC-MS/MS extracted ion chromatograms of a blank human urine sample (a) and a urine sample fortified with ZEN, ZAN, α-ZEL, β-ZEL, α-ZAL and β-ZAL at 0.2 ng mL^{-1} (b).

Fig. 4 Chromatograms of a naturally contaminated human urine sample before (a) and after (b) β-glucuronidase hydrolysis (3.68 ng/mL of ZEN, 2.64 ng/mL of α-ZEL and 1.32 ng/mL β-ZEL after enzyme hydrolysis).

Table 1. MRM transitions of the analytes.

Analyte	Precursor	Quantification ion	CV/CE[a]	Confirmation ion	CV/CE[a]	Ion ratio
ZEN	317.1	175.0	20/24	130.9	20/32	0.73
ZAN	319.1	275.0	48/22	205.1	48/22	0.61
α-ZEL	319.1	159.8	2/30	174.1	6/20	0.75
β-ZEL	319.1	159.8	36/28	174.1	36/26	0.88
α-ZAL	321.1	277.0	6/20	303.1	60/22	0.37
β-ZAL	321.1	303.1	26/20	277.3	46/28	0.89
^{13}C-ZEN	335.2	185.0	20/24	139.9	20/32	0.73

[a] CV, cone voltage (V); CE, collision energy (eV)

Table 2. Sensitivity, extraction recovery and matrix effect of the method

Analyte	R_E (Extraction recovery, %)	Matrix Effect (%)	R_A (Apparent recovery, %)	LOQ (ng mL^{-1})	LOD (ng mL^{-1})
ZEN	94.1	85.2	80.2	0.05	0.02
ZAN	100	81.8	81.8	0.1	0.03
α-ZEL	99.9	78.1	78.0	0.13	0.04
β-ZEL	111	84.2	93.5	0.2	0.06
α-ZAL	107	76.8	82.2	0.13	0.04
β-ZAL	116	77.2	89.6	0.07	0.02

Table 3. Accuracy and precision of the method

Analyte	Spiked level (ng mL^{-1})	Measured value (ng mL^{-1})	R_M (Method recovery, %)	RSD (%)	
				Intra-day (n=6)	Inter-day (n=18)
ZEN	0.5	0.48	95.9	6.9	8.5
	1	0.95	94.8	2.9	3.6
	5	4.58	91.7	1.2	2.7
ZAN	0.5	0.48	95.2	6.0	10.3
	1	0.88	87.9	3.9	6.0
	5	4.62	92.4	2.4	4.5
α-ZEL	0.5	0.45	90.4	3.1	8.2
	1	0.91	90.8	4.3	4.3
	5	4.64	92.9	3.3	5.9
β-ZEL	0.5	0.50	100	3.8	6.7
	1	0.92	91.6	3.8	3.9
	5	5.00	100	3.9	4.2
α-ZAL	0.5	0.50	100	4.3	10.7
	1	0.92	91.7	3.7	8.2
	5	4.65	93.0	1.8	4.1
β-ZAL	0.5	0.46	92.6	4.2	8.6
	1	0.96	95.7	3.2	5.9
	5	4.93	98.6	3.9	5.4

Table 4. Total ZEN and its metabolites detected in 301 human urine samples

Compound	>LOD (n)	Positive (%)	>LOQ (n)	Mean (ng mL^{-1})	Median (ng mL^{-1})	Range (ng mL^{-1})
ZEN	214	71.10	185	0.24	0.15	<LOQ ~ 3.7
ZAN	3	1.00	2	0.017	0.015	<LOQ ~ 0.52
α-ZEL	12	3.99	3	0.035	0.020	<LOQ ~ 2.6
β-ZEL	66	21.93	19	0.082	0.030	<LOQ ~ 2.1
α-ZAL	ND	ND	ND	ND	ND	ND
β-ZAL	ND	ND	ND	ND	ND	ND

ND: not detected (<LOD). For the calculation of means and medians, toxin concentrations <LOD were set to LOD/2; and toxin concentrations between LOD and LOQ were set to LOQ/2 of the respective compound.

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613 **Table 5. Total ZEN and its metabolites by gender and age groups**

	Compound	Positive n (%)	Mean (±SD) (ng mL^{-1})	Median (ng mL^{-1})	Range (ng mL^{-1})
Gender	Male (n=107)				
	ZEN	71 (66.4%)	0.19 (±0.25)	0.11	ND ~ 1.65
	ZAN	1 (0.9%)	0.015 (±0.003)	ND	ND ~ <LOQ
	α-ZEL	2 (1.9%)	0.030 (±0.099)	ND	ND ~ 1.04
	β-ZEL	18 (16.8%)	0.052 (±0.066)	ND	ND ~ 0.45
	Female (n=194)				
	ZEN	144 (74.2%)	0.27 (±0.39)	0.17	ND ~ 3.7
	ZAN	2 (1.0%)	0.018 (±0.036)	ND	ND ~ 0.52
	α-ZEL	10 (5.2%)	0.039 (±0.19)	ND	ND ~ 2.6
	β-ZEL	48 (24.7%)	0.099 (±0.26)	ND	ND ~ 2.1
Age	Age ≤12 (n=67)				
	ZEN	47 (70.1%)	0.27 (±0.49)	0.17	ND ~ 3.7
	ZAN	1 (1.5%)	0.016 (±0.004)	ND	ND ~ <LOQ
	α-ZEL	5 (7.46%)	0.072 (±0.33)	ND	ND ~ 2.6
	β-ZEL	12 (17.91%)	0.14 (±0.39)	ND	ND ~ 2.1
	12< Age ≤18 (n=36)				
	ZEN	32 (88.9%)	0.45 (±0.44)	0.35	ND ~ 2.4
	ZAN	1 (2.8%)	0.029 (±0.084)	ND	ND ~ 0.52
	α-ZEL	1 (2.8%)	0.021 (±0.008)	ND	ND ~ <LOQ
	β-ZEL	17 (47.2%)	0.11 (±0.18)	ND	ND ~ 0.92
	18< Age ≤65 (n=135)				
	ZEN	93 (68.9%)	0.21 (±0.27)	0.12	ND ~ 1.6
	ZAN	1 (0.7%)	0.021 (±0.008)	ND	ND ~ <LOQ
	α-ZEL	6 (4.4%)	0.03 (±0.09)	ND	ND ~ 1.0
	β-ZEL	31 (23.0%)	0.07 (±0.1)	ND	ND ~ 0.69
	Age >65 (n=63)				
	ZEN	23 (36.5%)	0.14 (±0.18)	0.07	ND ~ 0.89
	ZAN	0	ND	ND	ND
	α-ZEL	0	ND	ND	ND
	β-ZEL	6 (9.5%)	0.04 (±0.04)	ND	ND ~ 0.29

614 ND: not detected (<LOD). For the calculation of means, concentrations <LOD were set to LOD/2; and
615 concentrations between LOD and LOQ were set to LOQ/2 of the respective compound.

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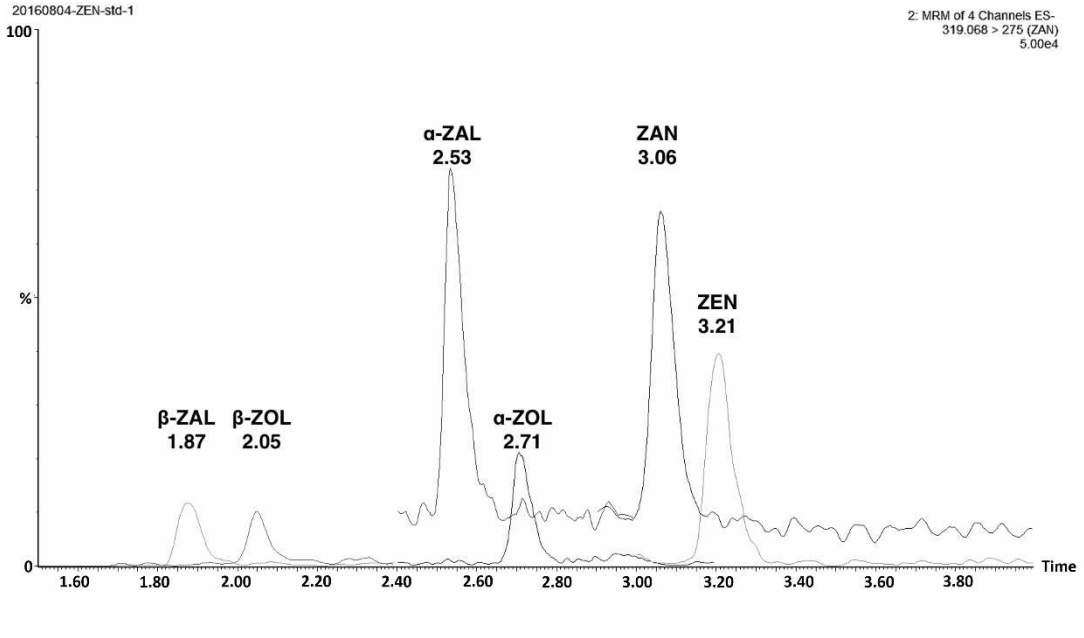
621 **Table 6. Occurrence of ZEN and its metabolites in human urine.**

Countries	N	Positive	Means/medians (ranges, ng/mL)	References
		Direct approaches		
Germany	101	4 (ZEN-14-GlcA)	<LOQ	[38]
Bangladesh	95	0	<LOD	[39]
Germany	50	0	<LOD	
Haiti	142	4 (α-ZEL)	1.46±1.02 (0.52-2.49)	
Belgium	40	4	(<LOD-12.6)	[36]
Belgium	32	0	<LOD	[45]
Belgium	239	1 (α-ZEL)	5.0	[46]
		2 (β-ZEL-14- GlcA)	0.6, 1.0	
	155	0	<LOD	
Cameroon	220	8 (ZEN)	0.97 (0.65-5.0)	[47]
		9 (α-ZEL)	0.98 (0.26-1.3)	
		18 (β-ZEL)	1.52 (0.02-12.5)	
Cameroon	145	4 (ZEN)	0.22 (<LOD-1.42)	[48]
		2 (α-ZEL)	<LOQ	
		4 (ZEN-14-GlcA)	0.81 (3.38-31)	
Thailand	60	0	<LOD	[49]
Nigeria	120	1 (ZEN)	0.3	[50]
		8 (ZEN-14-GlcA)	9.5 (<LOD-44.5)	
		Indirect approaches		
Italy	52	52 (ZEN)	0.057±0.023 (LOQ-0.120)	[51]
		52 (α-ZEL)	0.077±0.027 (LOQ-0.176)	
		51 (β-ZEL)	0.090±0.014 (<LOQ-0.135)	
Sweden	252	92 (ZEN)	0.03±0.06 (0.007-0.42)	[52]
		53 (α-ZEL)	0.03±0.13 (0.029-1.83)	
		45 (β-ZEL)	0.02±0.09 (0.054-1.33)	
Germany	13	13 (ZEN)	0.031±0.023 (0.007-0.09)	[53]
		6 (α-ZEL)	0.016±0.019 (<LOD-0.075)	
		3 (β-ZEL)	0.008±0.006 (<LOD-0.021)	
	12	12 (ZEN)	0.042±0.026 (0.007-0.09)	
		4 (α-ZEL)	0.015±0.015 (0.01-0.04)	
		2 (β-ZEL)	0.009±0.010 (0.01-0.04)	
South Africa	54	54 (ZEN)	0.204±0.456 (0.012-3.15)	[54]
		50 (α-ZEL)	0.247±0.590 (0.009-3.72)	
		40 (β-ZEL)	0.244±0.820 (0.016-5.94)	
The US	163	90 (ZEN)	1.82±4.80 (0.05-33.12)	[32]
		35 (α-ZAL)	0.25±0.13 (0.02-0.57)	
		60 (α-ZEL)	0.63±1.87 (0.003-10.69)	
		39 (β-ZEL)	0.35±0.23 (0.05-1.10)	
		17 (β-ZAL)	0.29±0.15 (0.04-0.60)	

		29 (ZAN)	0.33±0.59 (0.07-3.31)	
		128 (total)	1.86±5.73 (0.03-48.22)	
Tunisia	42	1 (ZEN)	<LOQ	[34]
		8 (α-ZAL)	(<LOQ-3.17)	
		1 (β-ZAL)	<LOQ	

Fig. 1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5.00e4
100
%
0
α-ZAL
2.53
ZAN
3.06
ZEN
3.21
β-ZAL
1.87
β-ZOL
2.05
α-ZOL
2.71
1.60
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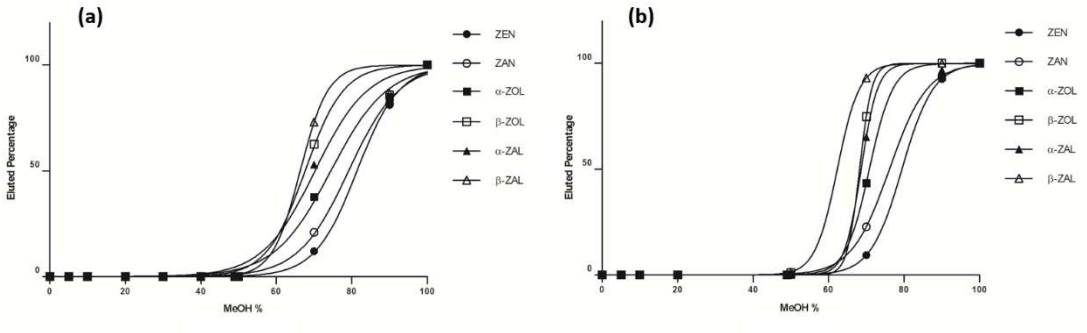
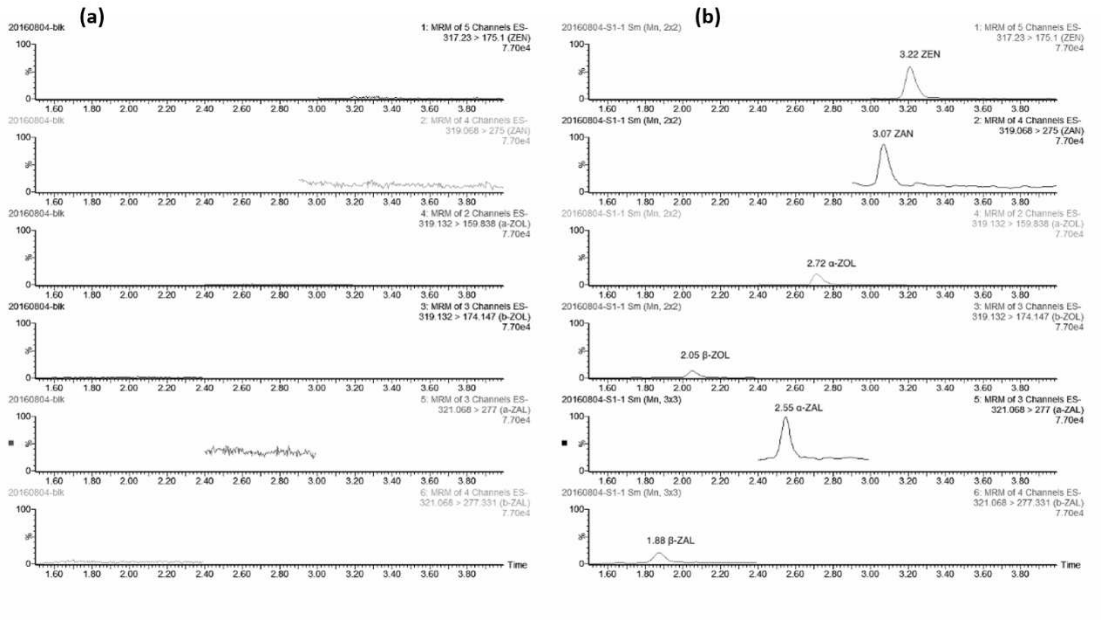
Time

Fig. 2


(a)
(b)
Eluted Percentage
MeOH %
ZEN
ZAN
α-ZOL
β-ZOL
α-ZAL
β-ZAL

662 **Fig. 3**



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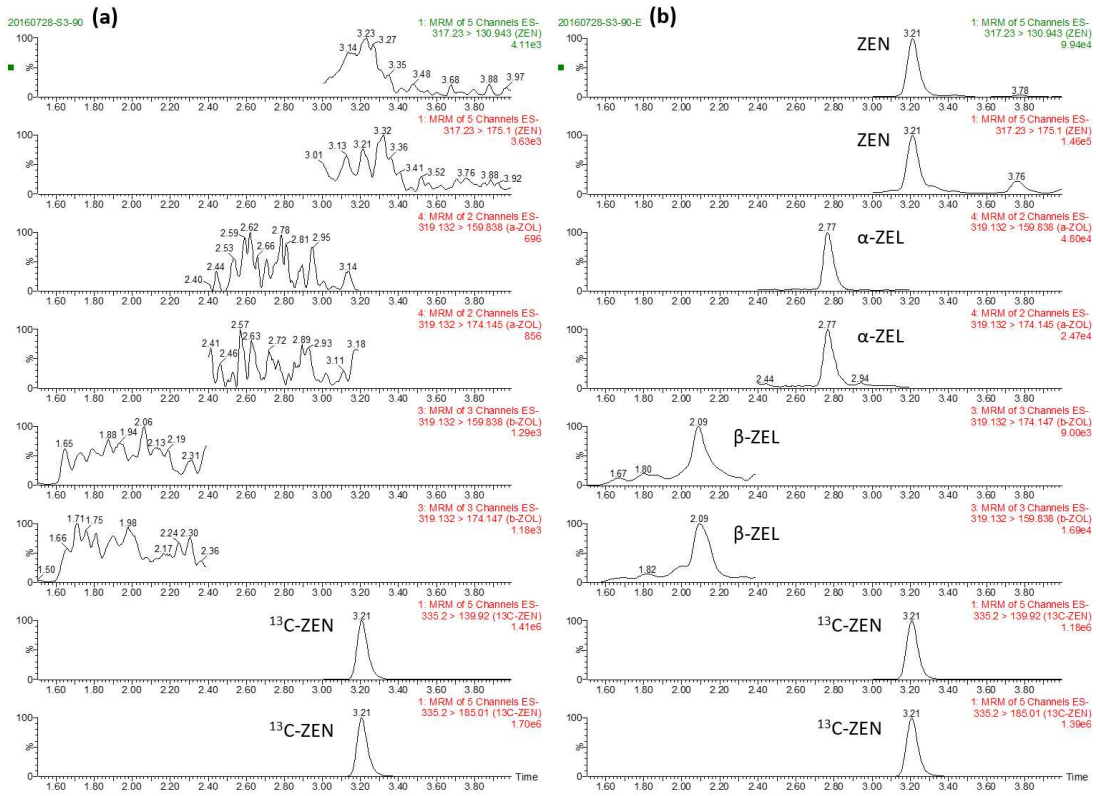
667 **Fig. 4**



668