

Methods for analysis of citrinin in human blood and urine

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Abstract Citrinin (CIT), produced by several *Penicillium*, *Aspergillus*, and *Monascus* species, has been detected as contaminant in feeds, grains, and other food commodities. CIT can co-occur with ochratoxin A (OTA), a mycotoxin also known for its nephrotoxicity, and this raises concern regarding possible combined effects. But, in contrast to OTA, data on CIT contamination in foods for human consumption are scarce, and CIT biomonitoring has not been conducted so far due a lack of suitable methods for human specimen. Thus, it was the aim of the present study to develop sensitive methods for the analysis of CIT in human blood and urine to investigate human exposure. To this end, we assessed different methods of sample preparation and instrumental analysis for these matrices. Clean-up of blood plasma by protein precipitation followed by LC–MS/MS-based analysis allowed robust detection of CIT (LOD 0.07 ng/mL, LOQ 0.15 ng/mL). For urine, sample clean-up by an immunoaffinity column (CitriTest®) proved to be clearly superior to SPE with RP₁₈ material for subsequent analysis by LC–MS/MS. For CIT and its metabolite dihydrocitrinone (HO-CIT), the LOD and LOQ determined by external calibration curves in matrix were 0.02 and 0.05 ng/mL for CIT, and those for HO-CIT were 0.05 and 0.1 ng/mL urine. The newly developed method was applied in a small pilot study: CIT was present in all plasma samples from 8 German adults, at concentrations ranging from 0.11 to 0.26 ng/mL. The molar (nM)

concentrations of CIT are similar to those measured for OTA in these samples as a result of dietary mycotoxin intake. CIT was detected in 8/10 urines (from 4 adults and 6 infants) in a range of 0.16–0.79 ng/mL, and HO-CIT was present in 5/10 samples at similar concentrations. Thus, CIT is excreted in urine as parent compound and also as metabolite. These first results in humans point to the need for further studies on CIT exposure.

Keywords Biomonitoring · Citrinin · Dihydrocitrinone · Plasma · Urine · IAC · LC–MS/MS

Introduction

Citrinin (CIT) is produced by several fungal species of the genera *Penicillium*, *Aspergillus*, and *Monascus*, and this mycotoxin has been detected as a natural contaminant in grains, foods, feeds and other commodities (Frank 1992; Xu et al. 2006). CIT is known to exert nephrotoxic effects in several animal species, and it has been implicated as a potential causative agent in human endemic Balkan nephropathy together with ochratoxin A (OTA), another nephrotoxic mycotoxin (IARC 1986; Petkova-Bocharova et al. 1991). This view is supported by the co-occurrence of CIT and OTA in foods from this region (Flajs and Peraica 2009; Vrabcheva et al. 2000). Both mycotoxins have been detected also in grains in Germany (Schmidt et al. 2003) and in breakfast cereals in France (Molinie et al. 2005).

Yet, compared with reports and surveys on OTA contamination of foods and feeds, there are relatively few data on the occurrence of CIT in commodities intended for human consumption (Table 1 in Flajs and Peraica 2009; Xu et al. 2006). This is due to the fact that there are presently no legal requirements to measure CIT in food and feed (EFSA

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2012) and because CIT analysis in these matrices is not trivial (Schmidt et al. 2003; Xu et al. 2006). On the other hand, there is a clear need to assess human exposure to CIT, also in the light of possible combined toxic effects of CIT and OTA (EFSA 2012; Flajs and Peraica 2009; Frank 1992).

In principle, human exposure to mycotoxins can be assessed by two approaches: analysis of levels in foods combined with food intake values and/or by measuring levels in blood or urine samples (biomonitoring). For OTA, such data are available for many European countries (EC/SCOOP 2002) and document that dietary exposure to this mycotoxin is widespread in the general population. OTA is frequently detected in blood (plasma or serum samples) from people in many countries and show some variation for different geographical locations and often with higher OTA blood levels in people from endemic (BEN) versus non-endemic regions of the Balkan and in patients diagnosed with kidney disease (data reviewed in Aslam et al. 2012; Degen 2011; Duarte et al. 2011; Märtilbauer et al. 2009). More variable OTA levels were reported in urine samples (Duarte et al. 2011; Gilbert et al. 2001; Muñoz et al. 2010; Scott 2005). As urine sampling is non-invasive, OTA analysis in this matrix is a promising alternative as a biomarker of exposure, especially in infants, although the relationship at the individual level between dietary intake and urinary biomarker needs to be developed further, including temporal variations in the presented levels (Duarte et al. 2011).

In contrast, biomonitoring of CIT has not been conducted so far, probably due to a lack of suitable analytical methods. In his review, H. K. Frank pointed out that “reports on the occurrence of citrinin in the human organism are not available”. This is still true, except for a short report on the (qualitative) detection of CIT in one human urine, yet with no details on the origin of the human sample and the CIT concentration (Orti et al. 1986), and one positively tested urine when a new multi-mycotoxin method was applied to 40 samples (Ediage et al. 2012). Therefore, it was the aim of the present study to develop sensitive methods suitable for the analysis of CIT in human blood and urine.

Materials and methods

Reagents, biochemicals, and standards

Methanol, acetonitrile (ACN), and acetic acid (96 %) were obtained from Merck (Darmstadt, Germany). Solvents used as mobile phase were HPLC and LC-MS grade, respectively. Citrinin (CIT) (CAS 518-75-2) (5 mg, purity >98 %) was purchased from Sigma-Aldrich (Taufkirchen, Germany), and solutions of CIT prepared in methanol were

calibrated spectrophotometrically at 321 nm using the molar extinction coefficient $5,490 \text{ M}^{-1} \text{ cm}^{-1}$ (Molinie et al. 2005). Dihydrocitrinone (HO-CIT) (CAS 65718-85-6), a metabolite of citrinin (purity 98.9 %), was from AnalytiCon Discovery GmbH (Potsdam, Germany). Working standard solutions of CIT and HO-CIT (see Fig. 1 for structures) were prepared weekly from a stock solution of 200 μg CIT/mL ACN and 500 μg HO-CIT/mL ACN, by dilutions in methanol in a range of 1–100 ng/mL. CIT in methanol solutions showed some instability after 3-month storage at -20°C , evident as additional peaks in HPLC. This was much less pronounced, but in line with observations by Clarke et al. (2006) on degradation of CIT in aqueous methanolic solutions. Experience for HO-CIT is lacking.

Sample collection

In this pilot study, healthy volunteers were asked to provide blood and urine samples, after signing an informed consent sheet. Blood samples were collected in winter 2010 in Dortmund, Germany, from a group of 5 men and 4 women (ages ranged between 16 and 58 years). Plasma was obtained from whole blood by centrifugation at 2,380 relative centrifugal force (rcf) (Labofuge 400R Heraeus, Germany) and kept frozen at -20°C until analysis. In 2012, urine samples from 10 volunteers were collected: 4 male adults (20–58 years) and 6 infants (age below 2 years). Urine samples (20 mL) were centrifuged at 3,940 rcf and then frozen and stored at -20°C .

Enzymatic cleavage of conjugates

To account for the possible presence of phase II metabolites (i.e. glucuronide or sulphate conjugates), all urine and plasma samples were enzymatically treated with β -Gluc/ArylSult prior to further sample clean-up (Muñoz et al. 2010).

Sample extraction

Plasma samples

The method for blood plasma consisted of a protein precipitation step as follows: 1 mL plasma was mixed with

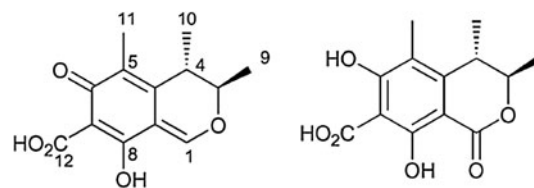


Fig. 1 Citrinin (*left*) and dihydrocitrinone (*right*)

acetonitrile (1:1, v/v). The sample was then centrifuged at 9,300 rcf (Centrifuge 5415D, Eppendorf, Germany) for 3 min. 1 mL of the upper layer was evaporated to dryness under a gentle N₂ stream at a temperature of 40 °C. The sample was then reconstituted in 300 µL methanol for chromatographic analysis.

Urine samples

Due to the complex urine matrix and probably low concentrations of analytes, sample clean-up was considered necessary. Thus, for the analysis of CIT, two clean-up methods were tested: C₁₈ solid-phase extraction (SPE) and immunoaffinity column (IAC) extraction.

Solid-phase extraction C₁₈ (SPE): 5 mL urine was mixed with 5 mL of 1 mM acetic acid in water. The mixture was loaded onto a C₁₈ cartridge (LiChrolut® RP-18, 40–63 µm, 500 mg, 3 mL from Merck), previously conditioned with 2 mL methanol and 2 mL water. The column was rinsed with 5 mL water followed by 5 mL of methanol/water (1:4, v/v) and dried by passing air through it. CIT was eluted with 3 mL methanol. The eluate was evaporated to dryness under a stream of nitrogen at 40 °C, and the residue was dissolved in 500 µL methanol and filtered through a 0.45-µm-pore-size Teflon syringe filter prior to HPLC analysis.

Immunoaffinity column (IAC) extraction: With the aim to compare the SPE with another frequently used clean-up method for biological samples, IAC for CIT was used as clean-up step for urine samples. CitriTest® columns (Vicam®, Watertown, MA, USA) were used for the clean-up. Five mL human urine was diluted with 5 mL of 1 mM acetic acid in water; the sample was mixed in a rotary shaker for 15 min and then quantitatively loaded on the CitriTest® column at a flow rate of 1 drop/s. The column was washed twice with 5 mL distilled water, then CIT was eluted (flow rate 1 drop/s) from the column using 4 mL of methanol. The eluate was evaporated to dryness under a stream of nitrogen at 40 °C, and the residue was dissolved in 500 µL methanol. Thus, the enrichment factor was 10.

HPLC analysis with fluorescence detection

Sample extracts and standards were analysed using an HPLC system (Shimadzu, Germany), consisting of two LC-10AS pumps, RF-10Axl fluorescence detector, SIL-10ADvp auto-injector, CBM-20A controller, and Shimadzu LC solution software. A C₁₈ column Nucleodur® Sphinx EC 125/3 (3-µm material) from Macherey & Nagel (Düren, Germany) was used for the separation of the analytes. The injection volume was 40 µL, and the analysis was performed at a column temperature of 22 °C. The

mobile phases (flow rate 0.4 mL/min) were the following: phase A water/methanol/acetic acid (96 %) (69.5:30:0.5, v/v/v) and phase B methanol/acetic acid (96 %) (99.5:0.5, v/v). The stepwise gradient was 0–4 min 100 % A, 4.5–17.5 min 45 % A, 18–20 min 20 % A, re-equilibration 21–30 min 100 % A. The fluorescence detector was set at 330-nm excitation and 500-nm emission wavelengths (Reinhard and Zimmerli 1999). The retention time for CIT was 12.5 min (Fig. 2).

LC-MS/MS analysis

Liquid chromatography with tandem mass spectrometry was used to measure CIT and its metabolite HO-CIT in urine and plasma samples. Detection was performed with a 1,200-L Quadrupole MS/MS equipped with an electrospray ionization (ESI) source and a Prostar® Varian HPLC system and Varian MS Workstation version 6.9.1 data system (Agilent Technologies, Germany). The following settings were used: nitrogen as drying gas (19 psi), gas temperature 200 °C, and argon used as collision gas (2.0 mTorr). Chromatographic separation was carried out at 21 °C on a Nucleosil® 100-5 C18 HD 125 × 3 mm column with ammonium formate 1 mM in water (mobile phase A) and ammonium formate 1 mM in methanol (mobile phase B) as eluents in the following gradient: 0–1 min 20 % B, 1–3 min 20–75 % B, 3–11 min 75 % B, 11–12 min 75–100 % B, 12–14 min 100 % B (column wash), 14–14.5 min 100–20 % B, and from 14.5 to 20 min 20 % B (re-equilibration). The flow rate was 0.3 mL/min. ESI-MS/MS was executed by multiple reaction monitoring (MRM) in negative ion mode. The specific transitions of precursor ion and product ion were as follows: 249.0 → 204.7 *m/z* (loss of the carboxyl group) and 249.0 → 176.7 for CIT. The optimized collision energy (CE) was 15 and 19 eV, respectively. For HO-CIT, the transitions of precursor and product ions were 265.0 → 221.0 and 265.0 → 176.7 *m/z* with an optimized CE of 16 and 20 eV, in that order. The retention time for CIT and its metabolite HO-CIT was 7.3 and 6.8 min (Fig. 3).

Results and discussion

Validation parameters

Instrumental detection limit

Two methods of analysis were tested: HPLC with fluorescence detection and LC-MS/MS. They were compared with regards to the instrumental limits of detection, in other words, the lowest amounts of CIT that are detectable by two systems available in our laboratory. LC-MS/MS

Fig. 2 Chromatogram of CIT standard solution (100 ng/mL in methanol) analysed by HPLC with fluorescence detection

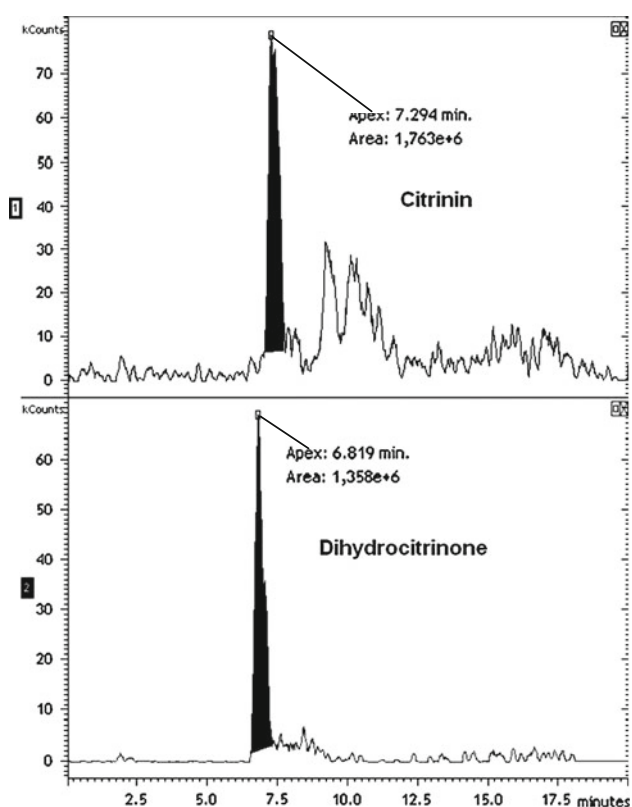
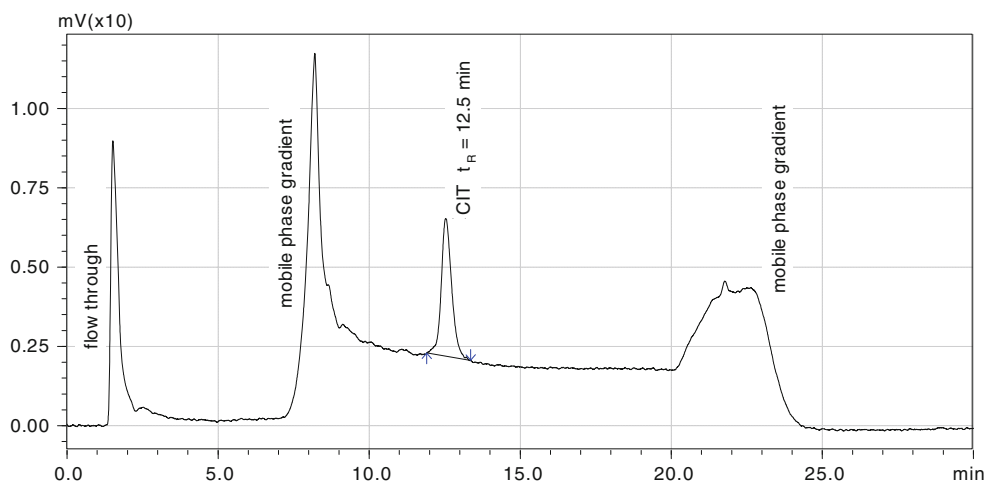


Fig. 3 Chromatogram of CIT (0.20 ng/mL, $t_R = 7.3$ min) and HO-CIT (1.12 ng/mL $t_R = 6.8$ min) in a human urine sample. The analysis was performed by using IAC sample preparation and LC-MS/MS

showed better results in terms of sensitivity than HPLC with fluorescence detection: the lowest amount measured with fluorescence by injecting 40 μ L was 400 pg, that is, 40 \times higher than the lowest amount measured with LC-MS/MS (10 pg). Since tandem mass spectrometric detection offered better analytical conditions, further analyses were performed using this system.

Matrix effect

Matrix effects can occur when molecules co-elute with the compound(s) of interest, affecting the ionization efficiency of the electrospray interface. The matrix effect was assessed by post-extraction addition method: this requires sample extracts with the analyte of interest added post-extraction compared with pure solutions prepared in mobile phase containing equivalent amounts of the analyte of interest. The difference in response (AUC) between the post-extraction sample and the pure solution divided by the response of the pure solution times 100 determines the degree of matrix effect in percentage occurring to the analyte in question under chromatographic conditions (Taylor 2005). When using human urine samples with non-detectable CIT, high ion suppression was observed after the C_{18} -SPE. On the other hand, a minimal suppression was registered when urine clean-up was done by immunoaffinity columns (Table 1).

Recovery

The recovery rate was tested for both the C_{18} -SPE and the IAC extraction method. Human urine samples (with non-detectable CIT) were spiked with known amounts of CIT (5, 10, or 200 ng/mL) and processed as described above. At higher concentrations, CIT was almost entirely recovered after C_{18} -SPE clean-up (95 and 97 %). However, at a low concentration (5 ng/mL), the recovery rate was only 10 %. This can be related to the strong matrix effect observed in the C_{18} -SPE post-extracted spiked samples, which makes the detection of CIT in human urine at low concentrations difficult.

A different situation was observed for clean-up of human urine with immunoaffinity columns. At a concentration of 5 ng CIT/mL urine, the recovery rate was higher than 70 %. Similar results were found even at much lower

Table 1 Calculation of matrix effects for CIT in urine cleaned up with C₁₈-SPE and IAC

	249 → 204 (<i>m/z</i>)	249 → 176 (<i>m/z</i>)
<i>Concentration 1 ng/mL</i>		
Pure solution	3,889,000 AUC	1,245,000 AUC
Post-extraction addition C ₁₈ -SPE	452,000 AUC	80,000 AUC
Post-extraction addition IAC	2,698,000 AUC	881,000 AUC
Matrix effect C ₁₈ -SPE	−88 %	−94 %
Matrix effect IAC	−31 %	−29 %
<i>Concentration 10 ng/mL</i>		
Pure solution	35,055,000 AUC	11,495,000 AUC
Post-extraction addition C ₁₈ -SPE	3,312,000 AUC	883,000 AUC
Post-extraction addition IAC	26,430,000 AUC	9,247,000 AUC
Matrix effect C ₁₈ -SPE	−91 %	−92 %
Matrix effect IAC	−25 %	−20 %

(100×) analyte concentrations. A summary of CIT recovery rates after immunoaffinity clean-up is shown in Table 2.

The outcomes of the recovery tests performed for both clean-up methods demonstrated the efficiency of the IAC over C₁₈-SPE at low analyte levels. An advantage of the IAC used (CitriTest[®]) is the cross-reactivity with the CIT-metabolite HO-CIT. A test performed at an OH-CIT concentration of 1 ng/mL ($n = 3$) results in a recovery rate of 106 ± 3 %. Therefore, the immunoaffinity clean-up was selected as the most suitable method for the extraction of CIT and HO-CIT from human urine samples.

Precision

The intra-assay repeatability was determined in urine with no background levels of CIT and HO-CIT. It was spiked with concentrations of 0.5 and 1.0 ng/mL. These spiked samples were analysed by LC-MS with IAC sample preparation ten and five times, respectively. Table 3 shows the values for the intra-assay repeatability.

Table 2 Recovery of CIT in human urine after clean-up with immunoaffinity columns

CIT concentration in urine (ng/mL)	Number of samples	Recovery ± RSD (%)
5	3	72 ± 35
1	3	77 ± 8
0.5	3	77 ± 12
0.1	3	66 ± 13
0.05	3	77 ± 25

For the intra-day repeatability, the same urine was applied. It was spiked with concentrations of 0.25 and 1.0 ng/mL and analysed as before. The samples were measured five times for both concentrations. Table 4 shows the data for the intra-day repeatability.

Accuracy

The relative recovery rates (Table 5) were calculated for all concentrations (0.25, 0.5, and 1.0 ng/mL) applied in the repeatability measurements in spiked urine samples with the method of IAC and LC-MS/MS.

Sensitivity of the method

Blood plasma samples: Since only unchanged CIT was found in blood of rats collected after administration of radiolabeled mycotoxin (Dunn et al. 1983), the metabolite HO-CIT was not measured in this matrix. The method for CIT involves a protein precipitation step followed by an enrichment of the analyte. Quantification was done by spiking plasma that shows no detectable CIT background. The LOD and LOQ of CIT were determined based on the lowest quantity of analyte that can be clearly distinguished (LOD) from the background ($S/N = 3$) or quantified (LOQ; $S/N \geq 6$). In plasma, the LOD for CIT was 0.07 ng/mL and LOQ was 0.15 ng/mL.

Urine samples: CIT and its metabolite HO-CIT were quantified by using external calibration curves in matrix. The calibration curves are linear in the range of 0.5 to 20 ng/mL for CIT and 1–20 ng/mL for HO-CIT (quantification range), with coefficients of determination (R^2) of 0.9978 and 0.9999 for CIT and HO-CIT, respectively. Spiked samples were used to define the minimum detectable level: the LOD and LOQ were determined based on the lowest quantity of analyte that can be clearly distinguished (LOD) from the background ($S/N = 3$) or quantified (LOQ; $S/N \geq 6$). For CIT, the LOD in urine was 0.02 ng/mL and the LOQ was 0.05 ng/mL. For HO-CIT, the LOD and LOQ were 0.05 and 0.10 ng/mL, respectively. These detection levels are considerably lower than those reported for CIT in urine (LOD 2.88 ng/mL; LOQ 5.76 ng/mL) by using a different sample clean-up method for subsequent multi-mycotoxin analysis (Ediage et al. 2012).

For urine, sample preparation with IAC (CitriTest[®]) and analysis of extracts with high-performance liquid chromatography and tandem mass spectrometric detection generated clean chromatograms, and no other interfering peaks were observed close to CIT. In relation to the validation parameters, the clean-up with IAC showed a low variation for CIT (RSD < 10 %) and the recovery levels were sufficiently high for analysis even at low analyte concentrations (Table 2). This is the first description of a

Table 3 Intra-assay repeatability for analysis of CIT and HO-CIT in urine by IAC and LC–MS/MS

Concentration ng/mL	0.5	0.5	1.0	1.0
Analyte	Citrinin	Dihydrocitrinone	Citrinin	Dihydrocitrinone
Mean (ng/mL)	0.53	0.53	1.05	0.92
SD (ng/mL)	0.13	0.11	0.10	0.09
CV (%)	25	21	9	10
<i>n</i>	10	10	5	5

Table 4 Intra-day repeatability for the assay of CIT and HO-CIT in urine by IAC and LC–MS/MS

Concentration (ng/mL)	0.25	0.25	1.0	1.0
Analyte	Citrinin	Dihydrocitrinone	Citrinin	Dihydrocitrinone
Mean (ng/mL)	0.30	0.28	0.95	0.89
SD (ng/mL)	0.05	0.05	0.16	0.21
CV (%)	15	19	17	23
<i>n</i>	5	5	5	5

method for the sensitive detection of CIT and its metabolite HO-CIT in human biological samples. The method was then applied in a pilot study to obtain first insights into CIT levels and metabolite occurrence in human urine and blood plasma samples.

Pilot study

CIT and its metabolite HO-CIT were determined in urines from a small cohort of 10 persons: 4 German adults and 6 Turkish infants. The age of the adults was between 20 and 60 years; all infants were below 2 years at the time of the sample collection. Urines were processed using the IAC-based method described above, and aliquots of all samples were also hydrolysed before analysis to account for the possible presence of glucuronides and/or sulphates. Quantification of the analytes was done by means of a calibration curve in matrix. Eight of ten urines were tested

Table 5 Relative recovery of CIT and HO-CIT in urine by IAC and LC–MS/MS

Concentration (ng/mL)	Intra-assay repeatability		Inter-day repeatability	
	Relative recovery (<i>r</i> %)	Range (%)	Relative recovery (<i>r</i> %)	Range (%)
Citrinin				
0.25	–	–	120	100–147
0.5	106	73–150	–	–
1	105	94–118	95	67–108
Dihydrocitrinone				
0.25	–	–	112	86–133
0.5	106	79–140	–	–
1	92	80–102	89	59–109

positive for CIT, and the metabolite was detected in 50 % of the samples. The results obtained in the human urines are summarized in Table 6.

These results show that CIT is mainly excreted with urine as parent compound and in some cases also as metabolite (HO-CIT). Dihydrocitrinone has been identified previously as a urinary metabolite of CIT in rats (Dunn et al. 1983). The analysis of human urine samples with and without enzymatic hydrolysis provided no evidence for the presence of conjugates in this matrix. This is in line with studies performed in rats, where the concentration of CIT in urine did not change (increase) after an enzymatic hydrolysis (Dunn et al. 1983).

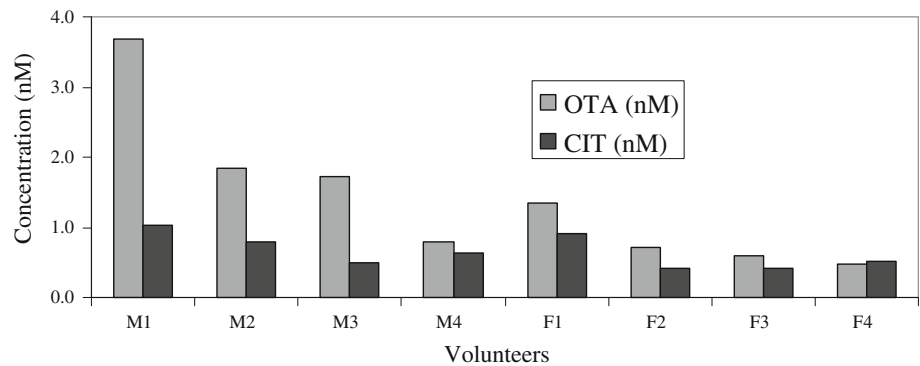
CIT was also analysed in blood samples of eight adult German volunteers (4 males and 4 females). These samples have been previously analysed by a validated method for

Table 6 Analysis of CIT and its metabolite HO-CIT in human urine samples from adults and infants

Volunteer	CIT		HO-CIT	
	Conc. (ng/mL)	Conc. (nM)	Conc. (ng/mL)	Conc. (nM)
Adult 1	<LOQ	<LOQ	0.28	1.05
Adult 2	0.05	0.20	0.20	0.75
Adult 3	0.05	0.20	<LOQ	<LOQ
Adult 4	0.07	0.28	0.34	1.28
Infant 1	0.09	0.36	<LOQ	<LOQ
Infant 2	0.04	0.16	<LOQ	<LOQ
Infant 3	0.20	0.80	1.12	4.21
Infant 4	<LOQ	<LOQ	0.15	0.56
Infant 5	0.07	0.28	<LOQ	<LOQ
Infant 6	0.05	0.20	<LOQ	<LOQ

Results are given in ng/mL and as nM (or nmol/L) concentrations

Fig. 4 CIT and OTA concentrations (nM) in blood plasma of German adults (*M* are males; *F* are females). 1 nM OTA = 0.40 ng/mL; 1 nM CIT = 0.25 ng/mL



the presence of OTA (Muñoz et al. 2010), and it was detected in all 8 samples. Now, with the newly developed method, CIT could be detected in all plasma samples from males and females, with values ranging from 0.10 to 0.25 ng/mL. The metabolite OH-CIT was not analysed in plasma, since only unchanged CIT was found in blood of rats in a kinetic study (Dunn et al. 1983). The CIT concentrations measured in human plasma were similar or somewhat lower than the OTA levels when expressed as nanomolar concentrations to account for differences in the molecular weights of CIT and OTA (Fig. 4). The results from this small survey are indicative of a frequent co-occurrence of two nephrotoxic mycotoxins (OTA and CIT) in blood of German adults, albeit at low nM levels.

Conclusions

A sensitive method for the analysis of citrinin in human urine and blood samples has been developed and validated. As shown in a small pilot study, the method is suitable for biological monitoring and allows detection of the mycotoxin (and its metabolite) in a high percentage of randomly collected samples. To the best of our knowledge, these are the first data on the frequent presence of citrinin and its main metabolite dihydrocitrinone in humans. It will be interesting to determine the enzymatic route(s) for the formation of this metabolite in mammalian species and its toxicity in relation to citrinin. Such studies are currently underway in our laboratory. Finally, additional studies are needed to identify the major dietary sources of human exposure to citrinin and on the resulting internal levels in different populations, along with studies on the co-occurrence of ochratoxin A.

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