

Ethylenebis(dithiocarbamates) and ethylenethiourea – Determination of ethylenethiourea in urine by LC-MS/MS

Biomonitoring Method – Translation of the German version from 2021

Keywords

ethylene bis(dithiocarbamates); ethylenethiourea; biomonitoring; urine; liquid chromatography; tandem mass spectrometry; LC-MS/MS

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Abstract

The working group “Analyses in Biological Materials” of the Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area developed and verified the biomonitoring method presented herein, which is used to determine the concentration levels of ethylenethiourea (ETU) in the urine of potentially exposed workers as well as non-occupationally exposed individuals in the general population with a quantitation limit of 0.5 µg/l.

ETU is extracted from urine with dichloromethane using solid-supported liquid extraction on diatomaceous earth columns. Eluates are blown to dryness, then the residues are reconstituted in the mobile phase and analysed by LC-MS/MS. Calibration is carried out with calibration standards prepared in pooled urine and treated in the same manner as the samples to be analysed. An isotopically labelled analogue of ETU (d₄-ETU) is used as an internal standard.

1 Characteristics of the method

Matrix	Urine
Analytical principle	Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS)

Parameter and corresponding hazardous substances

Hazardous substance	CAS No.	Parameter	CAS No.
Ethylenethiourea (Imidazoline-2-thione)	96-45-7		
Amobam (Diammonium ethylenebis(dithiocarbamate))	3566-10-7		
Mancozeb (Manganese zinc ethylenebis(dithiocarbamate))	8018-01-7		
Maneb (Manganese ethylenebis(dithiocarbamate))	12427-38-2	Ethylenethiourea (Imidazoline-2-thione)	96-45-7
Metiram (Zinc ammoniate ethylenebis(dithiocarbamate)- poly[ethylenebis(thiuramdisulfide)])	9006-42-2		
Nabam (Disodium ethylenebis(dithiocarbamate))	142-59-6		
Zineb (Zinc ethylenebis(dithiocarbamate))	12122-67-7		

Reliability data

Ethylenethiourea in urine

Within-day precision:	Standard deviation (rel.)	$s_w = 2.50\%$ or 1.89%
	Prognostic range at a concentration of 1 µg or 10 µg ETU per litre of urine (n = 10)	$u = 5.66\%$ or 4.28%
Day-to-day precision:	Standard deviation (rel.)	$s_w = 6.28\%$ or 4.83%
	Prognostic range at a concentration of 1 µg or 10 µg ETU per litre of urine (n = 6)	$u = 16.1\%$ or 12.4%
Accuracy:	Recovery rate (rel.)	$r = 119\%$
Detection limit:	0.25 µg ETU per litre of urine	
Quantitation limit:	0.5 µg ETU per litre of urine	

2 General information on ethylenebis(dithiocarbamates) and ethylenethiourea

Ethylenebis(dithiocarbamates) (EBDCs)

Amobam, Mancozeb, Maneb, Metiram, Nabam, and Zineb belong to the group of substances called ethylenebis(dithiocarbamate) pesticides and are applied primarily as fungicides, whereby they also possess insecticidal and herbicidal properties. In addition to their usage in agriculture, they are also used as slimicides in water-cooling systems and as scavengers in wastewater treatment (IPCS 1988).

Structurally, these substances are either ammonium, manganese, sodium, or zinc salts (Amobam, Mancozeb, Maneb, Nabam, Zineb) or polymeric metal-coordinated complexes (Metiram), which are – with the exception of Amobam and Nabam – fairly insoluble in both water and nonpolar solvents (IPCS 1988).

The substances decompose rapidly in water, with a half-life of just a few hours, leading to hydrolysis products such as ethylenethiourea (ETU), ethyleneurea, and ethylenebis(isothiocyanate) (IPCS 1988).

The metal-complexed ethylenebis(dithiocarbamates) are absorbed poorly both from the gastrointestinal tract and through the skin. The metabolic decomposition of EBDCs in mammals is complex. The main metabolite in mice and rats is ETU, and further metabolites include ethyleneurea, ethylenediamine, *N*-acetylene diamine, and ethylenebis(isothiocyanate). ETU also represents the primary metabolite in humans, however ETU also arises as a degradation product of EBDCs in the environment or as a production-related impurity of EBDCs, and can be taken in as such (Lindh et al. 2008). A generalised metabolism scheme of EBDCs is presented in Figure 1.

Mancozeb- and Metiram-based pesticides are permitted in Germany, although their most recent authorisations are slated to expire in December 2026 and January 2023, respectively (European Commission 2020, 2021). Furthermore, various Zineb-containing biocidal products (Product Type 21) may be purchased and used in Germany as a result of an ongoing decision-making process (marketability until December 2024) (European Commission 2014).

The systemic toxicity of EBDCs has been classified as relatively low, although there are concerns regarding ETU because of its occurrence as an impurity or as a metabolite due to teratogenic and goitrogenic effects which have been observed in experiments on animals (Lentza-Rizos 1990).

The Commission has designated Maneb with an “Sh” due to its skin-sensitising effect on humans (Greim 2001). The remaining EBDCs have not been evaluated by the Commission.

Ethylenethiourea (ETU)

Ethylenethiourea is used as a vulcanisation accelerator and antioxidant in the rubber industry, mainly in the production of polychloroprene (neoprene) (DECOS 1999; NTP 2011). ETU is furthermore used in electroplating baths, dyes, synthetic resins, and as a scavenger in wastewater treatment. Workplace exposure to ETU can occur in any of the aforementioned industries as well as in the handling and application of ethylenebis(dithiocarbamate)-containing products.

Potential exposure of the general population to ETU via migration from chloroprene rubber could not be conclusively proven (DK EPA 2004, 2012). Exposure in the general population can, however, occur as a result of consuming food products which have previously been treated with EBDCs. Residues of EBDCs and ETU are found in and on crops treated with EBDCs. The residue levels change during storage, processing, and cooking, as the parent compounds may be converted to ETU during these processes (IPCS 1988). Moreover, ETU can be found in cigarettes for which the tobacco had been treated with EBDCs (Aprea et al. 1996).

ETU, which is readily soluble in both water and oil, is rapidly absorbed (via inhalation as well as via dermal and oral routes) and metabolised. Up to 90% is eliminated with the urine (NTP 2011). Distribution of ETU in the body appears

to be fairly uniform with the exception of a relative accumulation in the thyroid gland. In mammals, plants, and the environment, ETU is either broken down to ethylene diamine (EDA), urea, carbon dioxide, and oxalic acid or is transformed to imidazole derivatives (IPCS 1988). ETU and its metabolites have been found to have a half-life of about 28 h in monkeys, 9–10 h in rats, and 5 h in mice (Rose et al. 1980). The elimination half-life of ETU in humans was estimated to be 17–23 h (Lindh et al. 2008).

While the International Agency for Research on Cancer (IARC) classified ETU in Group 3, namely “not classifiable as to its carcinogenicity to humans,” (IARC 2001), the substance was classified as “probably carcinogenic to humans” within the framework of the U.S. National Toxicology Program (NTP) (NTP 2011). The Commission has classified ETU in Carcinogen Category 3 (“suspected carcinogens”) (Greim 1998).

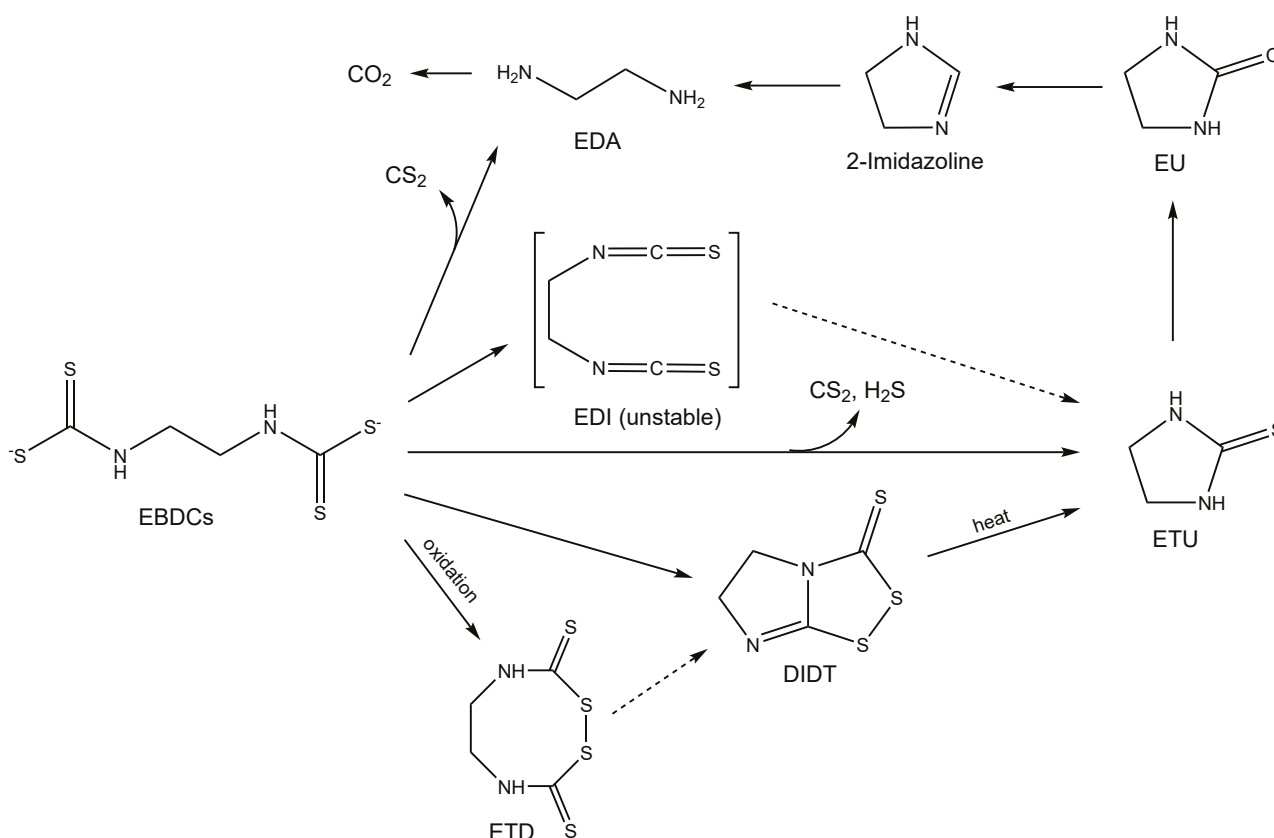


Fig. 1 Metabolism scheme of EBDCs (according to IPCS 1988) with EDA: Ethylenediamine, EDI: ethylene diisothiocyanate (unstable), DIDT: 5,6-dihydro-3H-imidazo[2,1-c]-1,2,4-dithiazole-3-thione, EU: ethyleneurea, ETU: ethylenethiourea, CS₂: carbon disulfide, and H₂S: hydrogen sulfide

3 General principles

This biomonitoring method is used to determine the concentration levels of ethylenethiourea (ETU) in the urine of potentially exposed workers as well as non-occupationally exposed individuals in the general population with a quantitation limit of 0.5 µg/l. ETU is extracted from urine with dichloromethane using solid-supported liquid extraction (SLE) on diatomaceous earth columns. Eluates are blown to dryness, then the residues are reconstituted in the mobile phase and analysed by LC-MS/MS. Calibration is carried out with calibration standards prepared in pooled urine and treated in the same manner as the samples to be analysed. An isotopically labelled analogue of ETU (d₄-ETU) is used as an internal standard. This method has been adapted from a method already published by Jones et al. (2010).

4 Equipment, chemicals, and solutions

4.1 Equipment

- HPLC system (e.g. Shimadzu UK Limited, Milton Keynes, United Kingdom)
- HPLC column: Gemini 5 μ C18, 150 \times 2 mm (e.g. Phenomenex Inc., Macclesfield, United Kingdom)
- Tandem mass spectrometer (e.g. Life Technologies Applied Biosystems API 3200 QTRAP, AB SCIEX Germany GmbH, Darmstadt, Germany)
- C18 Guard Column (e.g. Phenomenex Inc., Macclesfield, United Kingdom)
- Chem Elut columns, unbuffered, 3 ml (e.g. Agilent Technologies UK Ltd., Yarnton, United Kingdom)
- Nitrogen evaporator (e.g. Biotage AB, Uppsala, Sweden)
- Glass rimless culture tubes, 100 \times 16 mm (e.g. Fisher Scientific GmbH, Schwerte, Germany)
- 1.8-ml vials with micro inserts and crimp caps with rubber septa (e.g. Chromatography Direct Ltd., Runcorn, United Kingdom)
- Multipettes[®] with matching Combitips[®] (e.g. Eppendorf AG, Hamburg, Germany)
- Variably adjustable pipettes with matching pipette tips (e.g. Eppendorf AG, Hamburg, Germany)
- Various beakers and volumetric flasks (e.g. VWR International GmbH, Darmstadt, Germany)

4.2 Chemicals

Unless otherwise specified, all chemicals must be at least *pro analysi* grade.

- Ethylenethiourea (e.g. No. E057P025, Qmx Laboratories, Thaxted, United Kingdom)
- d₄-Ethylenethiourea (e.g. No. D-5348, C/D/N Isotopes Inc., EQ Laboratories GmbH, Augsburg, Germany)
- Acetonitrile (e.g. No. RH1015, Rathburn Chemicals (Mfg) Ltd., Walkerburn, United Kingdom)
- Formic acid (e.g. No. 533002, Merck KGaA, Darmstadt, Germany)
- Dichloromethane (e.g. No. RH1010, Rathburn Chemicals (Mfg) Ltd., Walkerburn, United Kingdom)
- Methanol (e.g. No. RH1019, Rathburn Chemicals (Mfg) Ltd., Walkerburn, United Kingdom)
- Ultra-pure water (e.g. Milli-Q plus VE System (> 18 M Ω), Merck KGaA, Darmstadt, Germany)

4.3 Solutions

- HPLC Eluent A (0.1% formic acid in methanol)
Pipette 1 ml of formic acid into a 1000-ml volumetric flask and make up to the mark with methanol.
- HPLC Eluent B (0.1% formic acid in ultra-pure water)
Pipette 1 ml of formic acid into a 1000-ml volumetric flask and make up to the mark with ultra-pure water.
- Mobile Phase (0.1% formic acid in 20% methanol)
Place 2 ml of the HPLC Eluent A in a 10-ml volumetric flask and make up to the mark with HPLC Eluent B.

4.4 Internal standard (ISTD)

- d_4 -ETU stock solution (10 g/l)
Approximately 100 mg of d_4 -ETU are weighed exactly into a 10-ml volumetric flask, which is then made up to the mark with acetonitrile.
- d_4 -ETU working solution (1 mg/l)
10 μ l of d_4 -ETU stock solution are pipetted into a 100-ml volumetric flask, which is then made up to the mark with ultra-pure water.

The stock and working solutions are stored in the refrigerator at 4 °C. Under these conditions, they are stable for at least three months (Montesano et al. 2007).

4.5 Calibration standards

- ETU stock solution (1 g/l)
Approximately 10 mg of ETU are weighed exactly into a 10-ml volumetric flask, which is then made up to the mark with methanol.
- ETU working solution (10 mg/l)
100 μ l of the ETU stock solution are pipetted into a 10-ml volumetric flask, which is then made up to the mark with methanol.
- ETU spiking solution (100 μ g/l)
100 μ l of the ETU working solution are pipetted into a 10-ml volumetric flask, which is then made up to the mark with methanol.

The ETU stock, working, and spiking solutions are stored in the refrigerator at 4 °C. Under these conditions, they are stable for at least three months (Montesano et al. 2007).

The calibration-standard solutions are freshly prepared in pooled urine each workday. The pooled urine is prepared using a mixture of different urines from non-smokers with no known exposure to any ethylenebis(dithiocarbamates) or ETU. This pooled urine is stored at –20 °C and passed through a folded filter prior to use. To prepare the calibration standards, the ETU spiking solution is mixed with pooled urine to a final volume of 2 ml, as indicated by the pipetting scheme given in Table 1. Processing of the calibration standards is carried out analogous to the samples to be analysed, as described in Section 5.

Tab. 1 Pipetting scheme for the preparation of calibration standards for the determination of ETU in urine

Calibration standard	Spiking solution [μ l]	Pooled urine [μ l]	ETU concentration [μ g/l]
1	0	2000	0
2	50	1950	2.5
3	100	1900	5
4	200	1800	10
5	300	1700	15
6	400	1600	20
7	500	1500	25

5 Specimen collection and sample preparation

5.1 Specimen collection

The urine samples are collected in urine cups and stored in the refrigerator at 4 °C until sample preparation. For long-term storage, the urine samples should be frozen at –20 °C. Under these conditions, they are stable for at least one year (Aprea et al. 1996).

5.2 Sample preparation

2 ml of the urine sample are pipetted into a sample vial and mixed with 50 µl of the working solution of the internal standard (d_4 -ETU working solution; 1 mg/l). After adding 1 ml of ultra-pure water, the sample solution is mixed and transferred onto the SLE cartridges. After ten minutes, 6 ml of dichloromethane are transferred onto the cartridge for the extraction of the analytes, and after ten more minutes, another 6 ml of dichloromethane are transferred. The eluting dichloromethane is completely collected and evaporated to dryness under a stream of nitrogen. The residue is then reconstituted in 100 µl of mobile phase (0.1% formic acid in 20% methanol). The measurement solution is pipetted into the micro insert of a 1.8-ml crimp-cap vial. The vial is capped and the sample is analysed by LC-MS/MS.

6 Operational parameters

Analysis is performed using a device configuration consisting of an HPLC system coupled with a tandem mass spectrometer (LC-MS/MS).

6.1 High-performance liquid chromatography

Analytical column:	5 µ C18 Gemini (150 × 2 mm)
Eluent:	A: 0.1% formic acid in methanol B: 0.1% formic acid in ultra-pure water
Elution:	isocratic (20% Eluent A)
Flow rate:	0.2 ml/min
Injection volume:	20 µl
Runtime:	5 min

At the beginning of the analytical run, samples are diverted away from the ion source using a six-port valve, and are redirected back to the ion source after one minute.

6.2 Tandem mass spectrometry

Ionisation mode:	Positive Electrospray Ionisation
Curtain gas:	25 psi
Collision gas:	medium
Ion-spray voltage:	3400 V
Desolvation temperature:	500 °C
Ion-source gas 1:	30 psi

Ion-source gas 2:	40 psi
Interface heater:	on
Parameter-specific settings:	see Table 2

Tab. 2 Parameter-specific settings for the determination of ETU in urine

Substance	Retention time [min]	Ion transition [<i>m/z</i>]		Collision Energy [V]
		Precursor ion	Product ion	
ETU	2.43	103	44.2	25
d ₄ -ETU	2.40	107	48.2	27

All settings are instrument-specific and must be adjusted individually by the user in addition to the daily optimisation routine of the MS/MS system. The parameters given are therefore intended only as cursory guidance.

7 Analytical determination

Of each of the samples processed according to [Section 5](#), 20 µl are injected into the LC-MS/MS system. The analyte is identified by its retention time and specific mass transitions (see [Table 2](#)). For this purpose, the retention times given in [Table 2](#) can only serve as a point of reference. Users must ensure proper separation performance of the column and any subsequent influence on the resulting retention behaviour of the analyte. A reagent blank (ultra-pure water instead of the urine sample) is included in each analytical run.

8 Calibration

For the purposes of calibration, calibration standards prepared as described in [Section 4.5](#) are processed in the same way as the samples (see [Section 5](#)) and analysed by LC-MS/MS (see [Sections 6](#) and [7](#)). The calibration curve is obtained by plotting the quotients of the peak heights of the analyte and ISTD against the spiked concentrations of the calibration standards. The calibration curve is linear under the described analytical conditions in the concentration range from 2.5 to 25 µg/l; the R² value should exceed 0.99. [Figure 2](#) shows a representative calibration curve for the determination of ETU in urine.

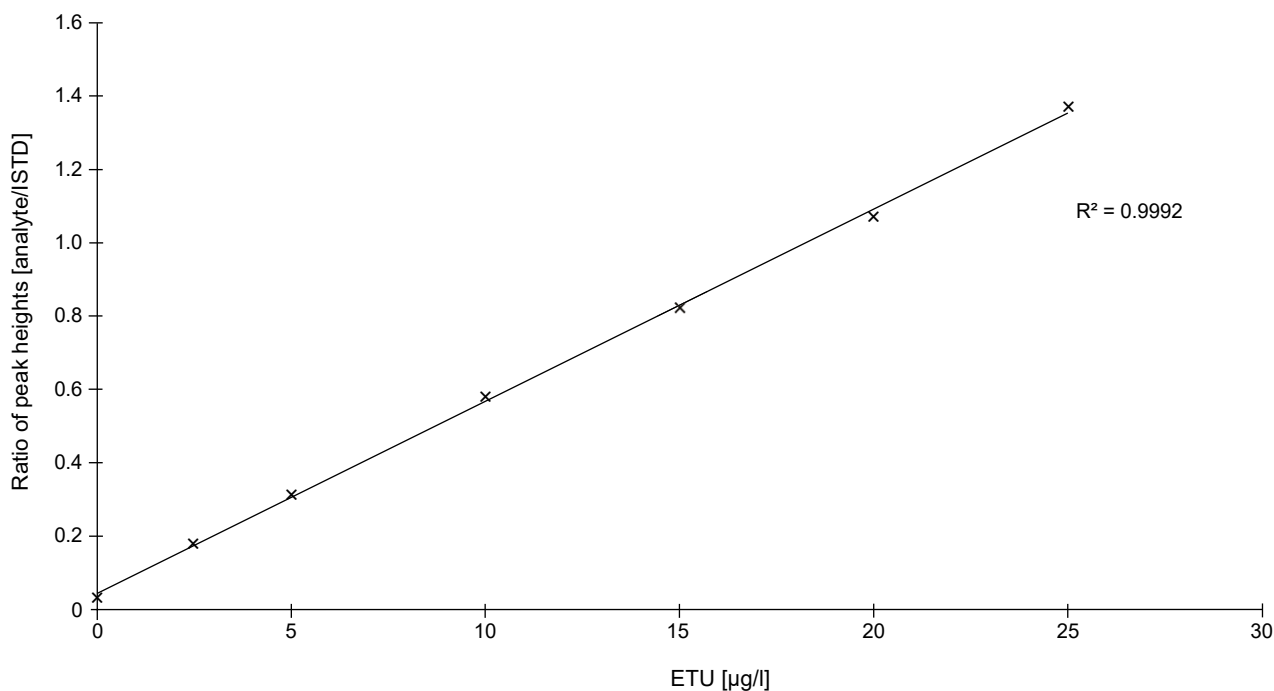


Fig.2 Calibration curve for the determination of ETU in urine

9 Calculation of analytical results

The analyte concentration in a urine sample is calculated by dividing the peak height of the analyte by that of the ISTD. With the aid of the corresponding calibration function (see Section 8), the respective analyte concentration can be calculated in µg/l of urine. If the analytical result lies above the calibration range, the relevant sample is diluted with ultra-pure water, reprocessed, and newly analysed.

10 Standardisation and quality control

Quality control of the analytical results is carried out as stipulated in the guidelines of the *Bundesärztekammer* (German Medical Association) and in a general chapter published by the Commission (Bader et al. 2010; Bundesärztekammer 2014). For precision control, each analytical run should include at least two quality-control samples with low (Q_{low}) and high (Q_{high}) analyte concentration levels.

To prepare the quality-control samples, pooled urine from individuals not exposed to ETU is used. The analyte concentration in the quality-control material should be within the relevant concentration range (see Section 9). Aliquots of these samples are frozen at -20°C and are included as quality-control samples in each analytical run.

11 Evaluation of the method

The reliability of this method was verified by comprehensive validation as well as by implementation and replication of the method in a second, independent laboratory.

11.1 Precision

Within-day precision was determined by spiking and analysing ten urine samples from individuals with no known exposure to ETU (1 µg/l). Furthermore, quality-control samples (10 µg/l) were processed and analysed ten times in parallel. The results are summarised in [Table 3](#).

Tab. 3 Within-day precision for the determination of ETU in urine (n = 10)

Analyte	Determined concentration [µg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
ETU	1.14	2.50	4.28
	8.72	1.89	5.66

Day-to-day precision was determined by processing and analysing the aforementioned ten individual urine samples (1 µg ETU/l) as well as ten quality-control samples (10 µg ETU/l) on six different days. The results are summarised in [Table 4](#).

Tab. 4 Day-to-day precision for the determination of ETU in urine (n = 6)

Analyte	Determined concentration [µg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
ETU	1.20	6.28	16.1
	8.63	4.83	12.4

11.2 Accuracy

The external verifier of this method investigated its accuracy and the influence of potential matrix effects using ten individual urines with creatinine concentrations in the range of 0.65 to 2.52 g/l. The urines were spiked with ETU at a concentration of 12.6 µg/l. Both spiked and unspiked urine samples were processed and analysed. The calculation of the relative recovery rates was carried out using the measured concentrations of the spiked urine, subtracting any background levels. The results of these investigations are presented in [Table 5](#).

Tab. 5 Mean relative recovery rates for the determination of ETU in urine (n = 10)

Analyte	Background level [µg/l]	Spiked concentration [µg/l]	Mean rel. recovery r [%]	Range [%]
ETU	0.14–4.11	12.6	98 ± 5	89.6–105

11.3 Matrix effects

Eight urine samples from persons with no known exposure to ETU, as well as ultra-pure water, were spiked with 10 µg/l of ETU and 25 µg/l of d_4 -ETU, processed, and analysed in triplicate. In addition, methanol was spiked with 10 µg/l of ETU and 25 µg/l of d_4 -ETU. This methanol sample was not processed, but was directly evaporated to dryness under a stream of nitrogen, and the residue was reconstituted in mobile phase and measured. The peak-height ratio of ETU to d_4 -ETU in the spike urine samples was compared with the peak-heights ratio of the methanol sample (see [Table 6](#)). A comparison of the recovery rate in urine (range 113–129%) with the recovery rate in the spiked water sample (102%) shows the presence of a matrix effect. In order to obtain accurate results, matrix-matched calibration in urine should be used for quantitation. Considering the varying creatinine concentrations of the urine samples as a gauge for the complexity of urine as a matrix, the complexity of the matrix exerts only a slight influence on the analytical results, as recovery varies only minimally at different creatinine concentrations (see [Table 6](#)).

Tab. 6 Matrix effects by the determination of ETU in urine (n = 3)

Sample	Creatinine [g/l]	Mean peak ratio ETU/d ₄ -ETU	Relative recovery rate <i>r</i> [%]
1	0.35	0.25 ± 0.01	113
2	0.70	0.25 ± 0.01	113
3	1.14	0.26 ± 0.00	121
4	1.52	0.27 ± 0.01	125
5	1.62	0.27 ± 0.00	123
6	1.92	0.28 ± 0.00	129
7	2.22	0.26 ± 0.00	117
8	2.63	0.27 ± 0.01	125

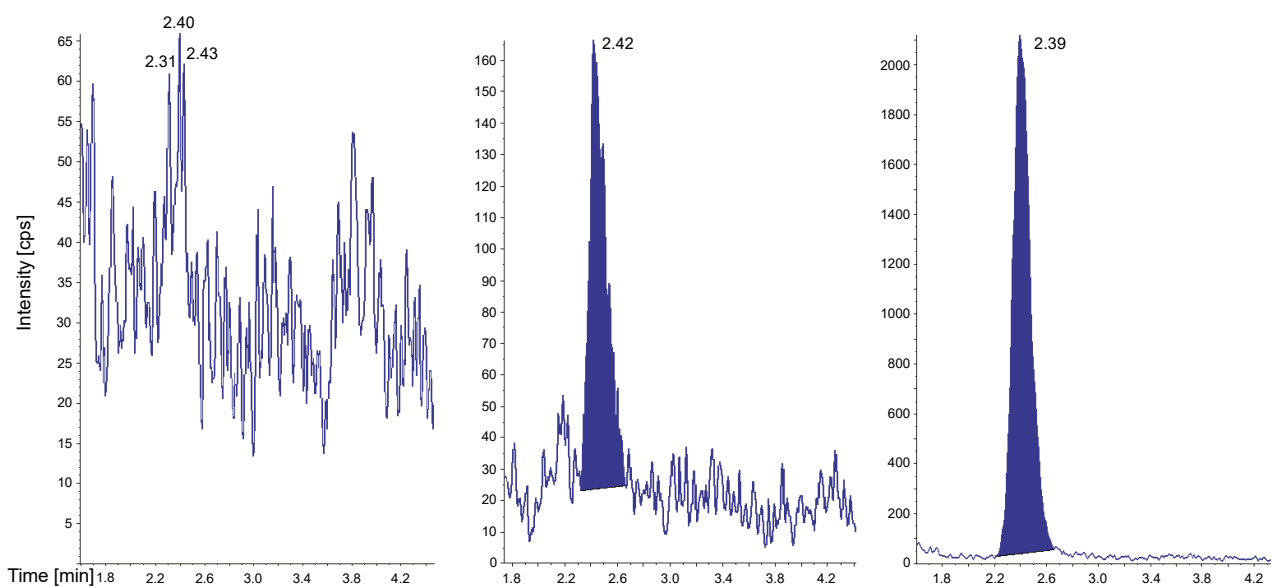
11.4 Limits of detection and quantitation

The detection limit was determined on the basis of a signal-to-noise ratio of 3 : 1. The limit of quantitation was similarly ascertained from the tenfold signal-to-noise ratio. The calculated values are shown in Table 7. Figure 3 shows chromatograms of an unspiked urine sample, of a urine sample spiked with ETU in the range of the detection limit, and of a urine sample spiked with 25 µg ETU/l.

The external verifier of the method determined the limits of detection and quantitation as stipulated by DIN 32645 (DIN 2008) and obtained values of 0.11 µg/l and 0.34 µg/l, respectively.

Tab. 7 Limits of detection and quantitation for the determination of ETU in urine

Analyte	Limit of detection [µg/l]	Limit of quantitation [µg/l]
ETU	0.25	0.5

**Fig. 3** Chromatograms of an unspiked urine sample, a urine sample spiked with ETU in the range of the detection limit (0.25 µg/l), and of a urine sample spiked with 25 µg ETU/l

11.5 Sources of error

ETU is readily photooxidised to ethyleneurea in the presence of UV-sensitisers (IPCS 1988). In order to avoid ETU degradation and any potential miscalculations, all stock and working solutions should be protected from direct sunlight.

12 Discussion of the method

The analytical method described herein allows for a simple, reliable, and sensitive determination of ETU concentration in urine. It is easily reproducible and linear in a concentration range of 2.5–25 µg/l ETU. The method presented here is similar to a method described by Jones et al. (2010) and achieves the same limit of detection (0.25 µg/l), but has some advantages. The method by Jones et al. (2010) used APCI (Atmospheric Pressure Chemical Ionisation), which requires much higher flow rates (0.8 ml/min) and therefore higher volumes of mobile phase. In contrast, the method described herein uses positive electrospray ionisation and thus requires, due to a flow rate of 0.2 ml/min and a simultaneously shorter analytical runtime, only a twelfth of the volume of eluent compared with the method of Jones et al. (2010). The reduced runtime of five minutes makes this method suitable even for laboratories with high sample throughput. The utilisation of tandem mass spectrometry leads to this method's high specificity and sensitivity.

The developers of this method measured the urine of 361 persons from the general population of the United Kingdom and found an average background exposure of 1.3 µg/g creatinine (≈ 1 µg ETU/l) (Jones et al. 2010).

Instruments used HPLC system (Shimadzu UK Limited, Milton Keynes, United Kingdom); HPLC column: Gemini 5 µ C18, 150 × 2 mm (Phenomenex Inc., Macclesfield, United Kingdom); tandem mass spectrometer (Life Technologies Applied Biosystems API 3200 QTRAP, AB SCIEX Germany GmbH, Darmstadt, Germany); C18 Guard Column (Phenomenex Inc., Macclesfield, United Kingdom)

Notes

Competing interests

The established rules and measures of the commission to avoid conflicts of interest (https://www.dfg.de/en/dfg_profile/statutory_bodies/senate/health_hazards/conflicts_interest/index.html) ensure that the content and conclusions of the publication are strictly science-based.

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