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Application of gas chromatography-mass spectrometry for the determination of urinary ethylenethiourea in humans

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Abstract

Ethylenethiourea (ETU) is a major metabolite of ethylenebisdithiocarbamate pesticides: a sensitive and specific assay for its determination in human urine is proposed below. ETU is extracted on a diatomaceous earth column using dichloromethane and derivatized with the mixture of *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide and *tert*-butyldimethyilsilyl chloride. The derivative is analyzed using GC/MS in the EI/SIM mode. The whole procedure is carried out in the presence of ethylenethiourea-d_4 as internal standard. The analytical features of the method are: high specificity, >90% recovery, range of linearity 0–200 μg/L, within- and between-run precision as coefficient of variation, <17 and <20%, respectively, limit of quantification 2 μg/L. In specimens stored in the dark at −20 °C ETU is stable for at least 6 months. The procedure was successfully applied to the biological monitoring of vineyard workers exposed to EBDTC and of a matched group of subjects from the general population.

Keywords: Urinary ethylenethiourea; Biological monitoring; Occupational exposure

1. Introduction

Ethylenethiourea (ETU) is both a degradation product of ethylenebisdithiocarbamate fungicides (EBDTC) [1–2] and a urinary metabolite of these chemicals in mammals [3]. While EBDTC are characterized by low acute and chronic toxicity, ETU showed a large spectrum of adverse effects in experimental animals, mainly concerning developmental toxicity and goiterogenic action, the latter related to the inhibition, then followed by a compensative increase, of the synthesis of thyroid stimulating hormones. In rodents the consequent thyroid hypertrophy might be followed by cancer. The evidence of effects on humans is less clear: a goitrogenic effect was reported in only two studies [4,5], while no teratogenic effects were observed. In 2001, the International Agency for Research on Cancer reviewed all the studies on cancerogenicity and classified ETU in group 3 (not classifiable as to its carcinogenicity in humans) based on inadequate evidence in humans, although sufficient evidence existed in experimental animals [6].

Exposure to EBDTC and/or ETU may occur in occupational settings, mainly in agriculture, during the use of EBDTC to protect crops, and in chemical plants, during their production. In occupational exposure, EBDTC and ETU enter the body through skin absorption and inhalation. Exposure may also occur in the general population, via ingestion of contaminated food (ETU was detected in vegetables and beverages) or via inhalation, in people living nearby areas treated with EBDTC [7–9].

For the assessment of occupational and non-occupational exposure to ETU and/or EBDTC in humans, the determination of ETU in urine was proposed [9–15]. In this context, concentrations ranging from <1 to hundreds of micrograms of ETU/L of urine were detected.

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Many analytical procedures for the determination of ETU have been published so far.

Techniques such as HPLC and GC were applied for the separation of ETU from other chemicals, and UV, MS, electrochemical and NPD detectors were used for its quantification (reviewed in [6,16]). The majority of these methods were developed for the determination of ETU in water or in complex matrices such as fruits and vegetables. Only a few of them are suitable for the quantification of the low level of ETU present in urine of occupationally and non-occupationally exposed subjects [10,17–19]. These methods use organic solvents to extract/purify ETU from urine, separate the analyte by liquid chromatography and detect it with a UV or MS detector. In our experience these procedures suffer from some drawbacks such as lack of an internal standard, use of a relatively large sample volume, low specificity (the LC/UV techniques), and the need for sophisticated equipment (the LC/MS technique).

In this work, an improved analytical assay suitable for the determination of a low level of ETU in human urine is presented. This assay uses a reduced amount of specimens, introduces an internal standard to control both the preparative and the analytical phases, and analyses ETU with a gas chromatograph coupled with a mass detector to ensure good and long lasting performance in separation and high specificity in the identification of the analyte. An application of the assay to the determination of ETU in agricultural workers exposed to EBDTC in vineyards and in a group of subjects from the general population is reported.

2. Experimental

2.1. General

2.1.1. Chemicals and standard preparation

ETU (>98%), ethylene-d_4-diamine (98%), *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (BSTFA, derivatization grade in sealed glass ampoule) and *tert*-butyldimethyilsilyl chloride (*t*-BuMe_2Si-Cl, >97%), ammonium chloride (NH_4Cl, 99.5%), dichloromethane (>99.9%), carbon disulfide (>99.5%) and iodine (99.8%) were from Sigma–Aldrich. Potassium fluoride (KF, >99%), acetonitrile (>99.9%) and methanol (>99.9) were from Riedel-de Haën. ETU was extracted from urine using diatomaceous earth column 3 mL (Chem Elut 1003, Varian). For purification and characterization of ETU-d_4 silica gel for flash chromatography (200–400 mesh) and silica gel thin layer chromatography plates 60 Å (layer thickness 250 μm, Merck) were used.

Acetonitrile was anhydrified on molecular sieve 4 Å (Carlo Erba Reagenti) before use. The derivatization mixture was obtained by daily mixing anhydrous acetonitrile, BSTFA and *t*-BuMe_2Si-Cl at the ratio 5:4:1 (v/v).

The standard solutions of ETU for the calibration curve were prepared at the concentration of 200, 100, 50, 25, 12.5, and 2.5 μg/L in urine. An unspiked sample of the same urine was kept as blank. The urine used for the preparation of these solutions was a pool of urine from non-smoking donors without occupational exposure to EBDTC and/or ETU. The solution of ehtylenethiourea-d_4 (ETU-d_4) to be used as internal standard was prepared at the concentration of 2.5 mg/L in water. The standard solutions of ETU and the internal standard solution were divided into small portions (about 10 mL) and stored in the dark at −20 °C.

2.2. Equipment

A 6890 Plus series gas chromatograph system, interfaced with a 5973 Network mass selective detector operating in the electron impact (EI) mode was obtained from Agilent. The gas chromatograph was equipped with a split-splitless injector operating in the splitless mode, with a CPSil 19 CB capillary column (30 m length, 0.25 mm internal diameter and 0.25 μm film thickness, Varian) and with a 7683 series autosampler (Agilent).

2.3. Synthesis of ethylenethiourea-d_4

Ethylenethiourea-d_4 (ETU-d_4), to be used as internal standard, was prepared reacting ethylene-d_4-diamine with carbon disulfide as previously described [20]. The raw product was purified by silica gel chromatography, and eluted with the mixture dichloromethane-methanol (95:5). ETU-d_4 was obtained as a white solid. The purified ETU-d_4 was characterized by thin layer chromatography on silica gel plate, eluting ETU-d_4 versus ETU with the mixture dichloromethane–methanol 95:5. The chemicals were developed as yellow-brown spots in the presence of I_2 vapours. Moreover, 1 mg purified ETU-d_4 was reacted with 100 μl of derivatisation mixture at 60 °C for 30 min. The reaction mixture was analysed by GC/MS using the conditions described below for the analysis of ETU, acquiring the mass spectra in the full scan mode (range *m*/*z* 40–400) (see Fig. 1).

2.4. Liquid/liquid extraction

To optimise the conditions for the liquid/liquid extraction of ETU from urine, the effects of the solvent's polarity and volume were investigated. With this aim, a solution of ETU in urine was dispersed on a diatomaceous earth column, as macroporous support. ETU was extracted using an increasing volume of pure dichloromethane, dichloromethane–isopropanol 95:5 (v/v) and dichloromethane–isopropanol 90:10 (v/v). The recovery of the extraction was estimated comparing the chromatographic signal obtained from a solution of ETU in urine with that obtained by direct analyses (i.e. without performing the extraction) of an aqueous solution of ETU at the same concentration.

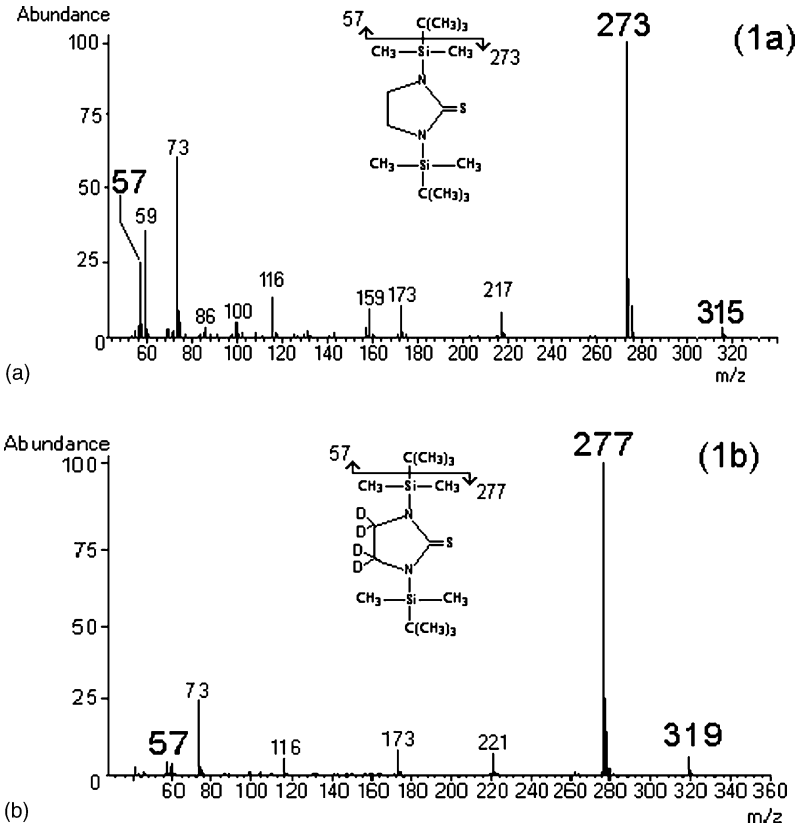

Fig. 1. Mass spectra of bis(*t*-BuMe$_2$Si)-ETU (a) and bis(*t*-BuMe$_2$Si)-ETU-d$_4$ (b) obtained in the EI mode.

2.5. Derivatization

The reaction of ETU and ETU-d$_4$ with the anhydrous acetonitrile, BSTFA, *t*-BuMe$_2$Si-Cl mixture 5:4:1 (v/v) to form the silyl derivatives was investigated at different temperatures. With this aim, 3 mL of an aqueous solution containing 100 μg/L ETU and 100 μg/L ETU-d$_4$ were evaporated and the residue added to 100 μL of derivatization mixture. The reaction was performed at 20, 60 and 80 °C and its kinetic followed by GC/MS starting from 0 to 60 h. The GC/MS analysis was performed in the conditions reported below for the analysis of ETU, acquiring the mass spectra in the full scan mode (range *m/z* 40–400) (see Fig. 1).

2.6. Sample preparation

The urine samples were left at room temperature until completely thawed. After shaking and waiting for a few minutes, 3 mL of urine surnatant were transferred in a glass vial containing 0.1 g of NH_4Cl and 1.5 g of KF. NH_4Cl and KF were used to adjust pH and ion strength, respectively. Then 0.1 mL of internal standard solution was added to the final concentration of 83.3 μg/L of ETU-d$_4$ in urine. The mixture was vigorously shaken to facilitate dissolution of salts and poured onto a diatomaceous earth column (3 mL, Chem Elut 1003, Varian). After urine percolation (about 5 min), 12 mL of dichloromethane were passed through the column and the organic solvent collected in a 20 mL glass vial. The extract was evaporated at 25–35 °C using a stream of nitrogen. The residue was dissolved with 1 mL (0.5 mL × 2) dichloromethane and transferred into a 1.8 mL glass vial. The solvent was gently evaporated at 25–35 °C using a stream of nitrogen and the residue was added to 0.1 mL of derivatization mixture. The vial was sealed with a plastic screw cap lined with a polyperfluoroethylene gasket and kept at 60 °C overnight. The residue, cooled at room temperature, was transferred to a conical insert and analysed as described below. Under these conditions ETU and ETU-d$_4$ reacted to give the bis-silanized derivatives bis(*t*-BuMe$_2$Si)-ETU and bis(*t*-BuMe$_2$Si)-ETU-d$_4$, respectively. The entire procedure allowed for preparation of about 24 samples/day.

2.7. Gas chromatography/mass spectrometry

The mixture containing the derivatized ETU and ETU-d$_4$ (1 μl) was injected into the chromatographic column through the injector liner kept at 250 °C. The oven temperature was kept at 150 °C for 1 min, then the temperature was increased to 240 °C at the rate of 20 °C/min. ETU and ETU-d$_4$ were eluted by keeping the oven at 240 °C for 5 min. The total run time was 10.5 min.

The MS detector, with the source temperature at 230 °C, was operating in the EI mode (70 eV) acquiring signal in the selected ion monitoring mode. The delay time was 4 min, the

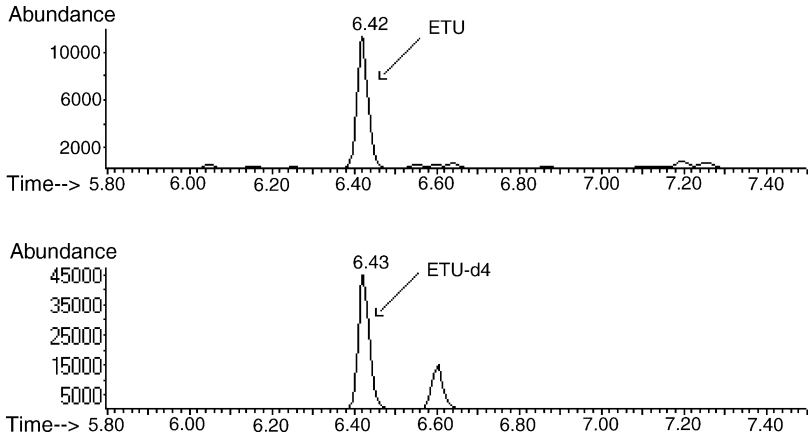

Fig. 2. Single ion mass-chromatograms obtained registering the ions *m*/z 273 and 277 for ETU and ETU-d_4, respectively, in a urine sample from a vineyard worker. The concentration of ETU in the sample was 17.5 μg/L, i.e. 9.2 μg/g creatinine.

dwell time was 100 ms. From 4 to 9 min the spectrometer was focused at ions *m*/z 273 and 277 $[M^{\bullet+} - C(CH_3)_3^{\bullet}]^+$ for bis(*t*-BuMe_2Si)-ETU and bis(*t*-BuMe_2Si)-ETU-d_4, respectively. Under the described conditions approximate retention times are as follows: bis(*t*-BuMe_2Si)-ETU = 6.42 min, bis(*t*-BuMe_2Si)-ETU-d_4 = 6.43 min (see Fig. 2).

2.8. Calibration, within- and between-run precision, accuracy

For calibration curve, the standard solutions containing ETU in urine at 200, 100, 50, 25, 12.5, and 2.5 μg/L and the blank were analyzed using the procedure outlined above. Least squares linear regression analysis was applied to estimate slope (*m*) of the function $y = mx$, where *y* is the ratio between the chromatographic peak area of the bis(*t*-BuMe_2Si)-ETU and the bis(*t*-BuMe_2Si)-ETU-d_4 derivatives and *x* is the ETU concentration in the sample (μg/L).

The analytical limit of detection (LOD), that is the lowest concentration whose analytical response can reliably differentiate from background level, was calculated as five times the ETU signal registered applying the assay to water samples (mean value of five replicates).

The lower limit of quantification (LOQ), that is the lowest concentration whose analytical response is identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80–120% [21], was calculated as five times the ETU signal registered applying the assay to blank urine samples.

The overall limit of detection and quantification of the assay were estimated dividing, respectively, the LOD and the LOQ by the mean level of urinary creatinine excreted in healthy subjects (1.2 g creatinine/L).

The within- and between-run precision of the assay (as coefficient of variation, CV%) was estimated by preparing solutions of ETU in urine at the theoretical concentrations of 2.5, 25 and 100 μg/L, and submitting each of them to the whole analytical procedure in quadruplicate on three different days [21–22].

2.9. Stability of ETU in urine

The stability of ETU in urine, kept in plastic tubes shielded from light with aluminum foil was evaluated through two experiments. The first, performed keeping samples at room temperature for 24 h, was intended to check the stability of ETU in specimens collected in the field and, for various reasons, not immediately refrigerated. For this experiment ETU was evaluated at time zero and after 24 h. The second, performed keeping samples at −20 °C, was intended to check the stability of ETU during storage of samples and standard solutions. For this experiment ETU in urine was evaluated several times over 6 months.

2.10. Urinary creatinine

Creatinine was determined using Jaffe's colorimetric method. In brief, creatinine was reacted with an aqueous solution of picrate (1% p/v) in NaOH (2.5 M). The quantification was based on the absorbance of the picrate complex at 512 nm, determined using a UV–vis spectrophotometer.

2.11. The field study

The urine samples were obtained from 13 agricultural workers using Mancozeb (EBDTC of Zn and Mg) in the treatment of vineyards and from 14 subjects belonging to the general population, without known exposure to EBDTC and/or ETU, matched with the workers for gender, residential area, tobacco smoking and alcohol intake. For workers, a spot urine sample was collected twice: before the beginning of the seasonal application of pesticides (pre-exposure sample) and after the exposure (post-exposure sample), prior to the next shift. For controls, a spot urine sample was collected once. All specimens were collected in Spring, in the morning, as second urine of the day. For urine collection, 15 mL polyethylene tubes shielded from light by aluminum foil were used. Samples were chilled, and delivered to the laboratory within

24 h of their collection. In the laboratory, the samples were kept at −20 °C until analysis.

3. Results

3.1. Ethylenethiourea-d_4

The characterization of ETU-d_4 by thin layer chromatography showed a single spot, coeluting with the commercial ETU, having a retention index of about 0.3. The GC/MS analysis of the ETU-d_4 after reaction with the derivatization mixture showed the co-presence of three species corresponding to the unreacted ETU-d_4 (t_r = 5.60 min), the mono-silanized derivative (*t*-BuMe$_2$Si)-ETU-d_4 (t_r = 6.24 min) and the bis-silanized derivative bis(*t*-BuMe$_2$Si)-ETU-d_4 (t_r = 6.46 min). The principal peak ions in the mass spectra were as follow. ETU-d_4: *m*/*z* 106 $[M]^{\bullet+}$ (100%); (*t*-BuMe$_2$Si)-ETU-d_4: *m*/*z* 220 $[M]^{\bullet+}$ (4%), 163 $[M^{\bullet+} - C(CH_3)_3^{\bullet}]^+$ (100%), 205 $[M^{\bullet+} - CH_3^{\bullet}]^+$ (4%); bis(*t*-BuMe$_2$Si)-ETU-d_4: 277 $[M^{\bullet+} - C(CH_3)_3^{\bullet}]^+$ (100%), 319 $[M^{\bullet+} - CH_3^{\bullet}]^+$ (7%) (for mass spectra see Fig. 1b). Based on these experiments the purity of ETU-d_4 was >99%.

3.2. Liquid/liquid extraction

Comparison of the solvents showed that their efficiency in the extraction of ETU in urine was similar. The extraction volume of 12 mL was the minimum volume that ensured the extraction of the analyte to be almost complete. Based on these results the liquid/liquid extraction was performed using 12 mL pure dichloromethane. Under this condition the recovery of the extraction was estimated to be higher than 90%.

3.3. Derivatization

The kinetic of derivatization of ETU and ETU-d_4 was characterized by the presence of three chromatographic peaks for each reactant. Based on the mass spectra they were identified as unreacted ETU/ETU-d_4, retention time ∼5.6 min, the mono-silanized derivative (*t*-BuMe$_2$Si)-ETU/ETU-d_4, retention time ∼6.2 min, and the bis-silanized derivative bis(*t*-BuMe$_2$Si)-ETU/ETU-d_4, retention time ∼6.4 min. For ETU, the principal peak ions in the mass spectra were as follows. ETU: *m*/*z* 102 $[M]^{+\bullet}$ (100%); (*t*-BuMe$_2$Si)-ETU: *m*/*z* 216 $[M]^{+\bullet}$ (4%), 201 $[M^{+\bullet} - CH_3^{\bullet}]^+$ (4%), 159 $[M^{+\bullet} - C(CH_3)_3^{\bullet}]^+$ (100%); bis(*t*-BuMe$_2$Si)-ETU: 315 $[M^{+\bullet} - CH_3^{\bullet}]^+$ (7%), 273 $[M^{+\bullet} - C(CH_3)_3^{\bullet}]^+$ (100%) (for mass spectra see Fig. 1a). For ETU-d_4, the principal peak ions in the mass spectra were as described above. Under this experiment the two chemicals behaved similarly, and for simplicity only the results of ETU are reported. In Fig. 3, the kinetic of the reaction performed at 60 °C is shown. The disappearance of ETU and the formation of its derivatives are summarized according to the abundance of the most representative ion for each species, i.e. *m*/*z* 102 for ETU, 159 for (*t*-BuMe$_2$Si)-ETU and 273 for bis(*t*-BuMe$_2$Si)-ETU. Soon after the beginning of the reaction the unreacted ETU disappeared and the mono-silanized derivative became the major species. The bis-silanized derivative slowly increased its presence and after 15 h the mono- and bis-silanized derivatives had the same abundance. Later on the bis-silanized derivative was the major chemical, but even at a reaction time of 58 h the mono-silanized derivative was still present.

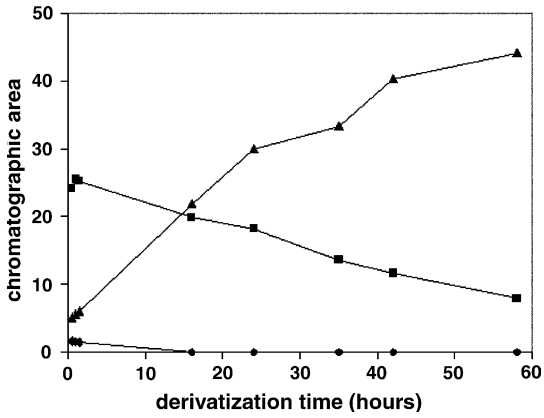

Fig. 3. Kinetic of the reaction of ETU with the derivatization mixture at 60 °C. On *Y*-axis is reported the chromatographic area of the peaks obtained registering the single ions *m*/*z* 102 for ETU (♦), 159 for *t*-BuMe$_2$Si-ETU (■) and 273 for bis(*t*-BuMe$_2$Si)-ETU (▲) and on *X*-axis is reported the reaction time.

Similar behaviour, but different reaction rates, were observed at 20 and 80 °C. While the reaction at 20 °C was extremely slow, the reaction at 80 °C allowed the bis-silanized derivative to be preponderant starting from a reaction time of 8 h. A drawback of operating at 80 °C was the evaporation of solvent from reaction vials.

Based on the results of these experiments, the bis(*t*-BuMe$_2$Si)-ETU was chosen as chemical to be monitored for the determination of ETU, and its most abundant ion *m*/*z* 273, corresponding to the loss of *tert*-butyl radical from the molecular ion, was selected as quantifier for further acquisition of mass chromatogram in the single ion monitoring mode. Similarly, the ion *m*/*z* 277 was chosen for monitoring the internal standard ETU-d_4 as bis(*t*-BuMe$_2$Si)-ETU-d_4. To operate the derivatization of ETU, a temperature of 60 °C was chosen as a compromise between reaction rate and solvent evaporation. Furthermore, although the reaction is not completed, a convenient reaction time of about 16 h or overnight was chosen.

3.4. Chromatographic separation

In Fig. 2, the single ion mass-chromatograms obtained registering the ions *m*/*z* 273 for ETU (above) and 277 for ETU-d_4 (below), from a urine sample of an agriculture worker, are reported. The analytes were univocally assigned based on their

Table 1
Summary of the linear regression parameters for the calibration curve and limits of detection for the determination of ETU in urine

Assay's parameters	Values
Dynamic range (μg/L)	0–200
Correlation coefficient (r)	0.998
Determinations (N)	14
Slope ± S.E.	0.013 ± 0.0001
Intercept ± S.E.	Forced thought zero
LOD (μg/L)	0.6
Limit of detection of the assay (μg/g creatinine)[a]	0.5
LOQ (μg/L)	2.0
Limit of quantification of the assay (μg/g creatinine)[a]	1.6

[a] Estimated based on a mean urinary creatinine excretion of 1.2 g/L.

retention time and mass to charge ratio. The concentration of ETU in the sample was 17.5 μg/L or 9.2 μg/g creatinine.

3.5. Calibration, within- and between-run precision, accuracy

Typical parameters for the calibration curve, LOD and LOQ for the determination of ETU in urine are reported in Table 1. In Table 2 are summarized the within- and between-run precision and the accuracy of the assay.

3.6. Stability of ETU in urine

Following this experiment it was found that ETU in urine samples kept in the dark at room temperature and at −20 °C was stable for 24 h, and for at least 6 months, respectively.

Table 2
Within- and between-run precision and accuracy for the determination of ETU in urine

	Theoretical concentration ETU (μg/L)		
	2.5	25	100
Day 1			
Mean (N = 4)	3.0	25.0	101.2
CV%[a]	16.5	14.9	7.7
% Theoretical[b]	120	100	101
Day 2			
Mean (N = 4)	2.7	24.4	98.7
CV%[a]	10.9	6.0	7.2
% Theoretical[b]	107	98	99
Day 3			
Mean (N = 4)	2.1	27.9	108.5
CV%[a]	6.9	5.2	1.7
% Theoretical[b]	85	111	109
Overall			
Mean (N = 12)	2.7	25.8	102.8
CV%[c]	19.7	10.5	6.9
% Theoretical[b]	108	103	103

[a] Within-run precision.
[b] Accuracy.
[c] Between-run precision.

3.7. The field study

In Table 3 are summarized the statistics of the excretion of urinary ETU in vineyard workers and in controls. None of the controls' and workers' pre-exposure samples had ETU levels higher than the limit of quantification of the assay. In workers' post-exposure specimens, 10 samples had urinary ETU higher than the limit of quantification of the assay, with a mean level of 10.5 μg/g creatinine.

4. Discussion

The present work describes an original analytical procedure based on GC/MS, useful to perform routine analysis of urinary ETU among subjects occupationally and non-occupationally exposed to EBDTC and/or ETU.

Our previous attempts to apply an HPLC/UV procedure for the determination of urinary ETU presented several drawbacks. Among them, the weak interaction between ETU and the reverse phase silica gel of the tested columns (e.g. C18 silica gel, 150–250 mm length, 4.0–4.6 mm internal diameter, 5 μm particle size), which implied scarce chromatographic performance, with short retention time, even using mobile phases mainly constituted by water, and quick loss of retentive capability with consequent interference between the peak of ETU and those of unknown impurities deriving from urine. This problem was particularly relevant when low ETU concentration had to be quantified. In fact, due to the scarce specificity of the UV detector a sure attribution of the chromatographic signal to ETU was not possible and the attempt to increase specificity by registration of the UV spectra via diode array detector failed, due to its low sensitivity. Further difficulty in the use of HPLC/UV technique was the identification of a suitable internal standard.

To overcome the problems encountered working in HPLC/UV, and therefore to increase specificity and sensitivity of the assay, we switched to GC/MS. Due to scarce volatility of ETU, the first step toward this technique was the identification of a suitable derivative, useful for GC analysis. Although in literature several derivatization protocols were proposed for GC analysis of ETU [23–29], our interest was focused on the use of silylating agents that could react with both the functional groups present in ETU: the secondary amide nitrogens and the sulfur of the thiocarbonyl moiety. Moreover, reaction mixture containing silylating agents did not need a purification step before introduction in the chromatographic column. The major drawback of this derivatization was the low reactivity of ETU functional groups vs. the silylating agents, due to the poor nucleophilicity of nitrogens and sulfur, since their electron pairs are delocalized among three tautomeric forms. In the light of this, a highly reactive silylating mixture containing BSTFA and *t*-BuMe$_2$Si-Cl was adopted. The study of the kinetic of the reaction at different temperatures (see Fig. 3), showed that both long reaction time and high temperature were necessary to completely

Table 3
Summary of statistics for urinary ETU excretion in 13 vineyard workers (pre- and post-exposure samples) and in 14 controls

Subjects (*N*)	Sampling time	Urinary ETU (μg/g creatinine)				
		Mean	S.D.	Median	Minimum	Maximum
Vineyard workers (13)	Pre-exposure	<1.6[a]	–	<1.6	<1.6	<1.6
	Post-exposure	10.5	11.3	8.5	<1.6	40.1
Controls (14)	–	<1.6	–	<1.6	<1.6	<1.6

[a] 1.6 μg/g creatinine is the limit of quantification the assay.

convert ETU to the bis-silanized derivative. Nevertheless a reaction temperature of 60 °C and a reaction time of 16 h or overnight were chosen, as a compromise between the reaction completeness and convenient conditions. In these conditions, a prevalence of bis-silylated derivative was obtained, with a significant co-presence of mono-silylated derivative. Although the reaction was incomplete, the introduction of the ETU-d_4 as internal standard ensured good performance to the assay, as shown in Tables 1 and 2. A complete conversion to bis-silanized derivative would further increase sensitivity of the assay. The molecular structure of this derivative was not univocally established, although attempts were made to elucidate its structure through the interpretation of mass spectra. Based on nucleophilicity of electron pairs on nitrogen and sulfur atoms a tentative formula with both nitrogen atoms derivatized is suggested (see Fig. 1a).

Among major advantages of the use of GC/MS technique are the great specificity and sensitivity of the assay: the identification of the bis-silanized derivative of ETU was based on both the acquisition of the single ion *m*/*z* 273, and on the retention time of the peak. These identification criteria are much stricter than those afforded by HPLC/UV. The single ion chosen is the most representative for abundance and is highly specific, being formed by loss of *tert*-butyl radical from the molecular ion of the bis-silanized derivative. The retention time of this peak was constant over time thanks to long lasting performance of the capillary chromatographic column used. Moreover, the adopted acquisition mode allowed elimination of the peaks deriving from the several interfering compounds present in the biological matrix. As a final consequence of the good specificity and sensibility obtained via GC/MS, the introduction of a simple sample preparation, performed by liquid/liquid extraction of a small volume of urine (3 mL), followed by derivatization, was feasible. Using the outlined conditions, the procedure was accelerated to prepare as many as 24 urine samples/day.

Finally, the use of MS detector allowed the introduction of ETU-d_4 as internal standard. This molecule was synthesized and purified, and the product obtained had a high isotopic purity, as requested for the quantification of low ETU levels. The use of this standard from the very beginning of the assay corrected many casual errors that may occur, especially during sample preparation, and ensured good precision of the assay, as shown in Table 2. The use of propylenethiourea (PTU) as internal standard was alternatively investigated. The performances of the entire procedure were very similar to those obtained using ETU-d_4. The advantage of using PTU, rather than ETU-d_4, is its commercial availability. A major drawback is that PTU itself is the metabolite of pesticides such as Propineb, so it may not be employed where this exposure takes place.

Due to previous studies in which the degradation of ETU, spontaneous or mediated by light and/or heat, was shown [30–31], the stability of ETU in urine samples was investigated. For this study, two conditions typically encountered in the field study and/or during sample storage were considered. The result of this study showed that ETU in urine samples shielded from light was pretty stable both at room temperature (at least 24 h) and at −20 °C (at least 6 months). This information is very useful for a safe management of samples.

So far the assay has been successfully applied to routine analysis of hundreds of urine samples. As example, typical levels found in agricultural workers exposed to EBDTC in vineyards during operations of mixing, loading, application and re-entry in Northern Italy, and in suitable groups of matched controls, are shown in Table 3. The correction for urinary creatinine was applied to raw data expressed as μg/L to take into account both dilution of urine and kidney activity. The collection of pre-exposure urine sample in workers and of urine sample in controls allowed us to obtain ETU levels in the absence of occupational exposure, to be used as reference values. Due to the lack of biological limit values for urinary ETU, these values represent terms of reference for comparison with urinary ETU levels in samples collected in workers after exposure. The urinary ETU levels found in the investigated subjects are in line with those reported in previous studies performed on agriculture workers and/or general population, obtained using analytical procedures based on HPLC/UV or LC/MS [9–15,19].

Based on the features of the above described analytical procedure, in terms of urine volume required, time necessary for performing the assay, sensitivity, specificity, precision and accuracy, we conclude that the present approach represents a useful tool for the biological monitoring of exposure to EBDTC and/or ETU through the determination of urinary ETU.

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