Detection and Quantification of Benzothiazoles in Exhaled Breath and Exhaled Breath Condensate by Real-Time Secondary Electrospray Ionization-High-Resolution Mass Spectrometry and Ultra-High Performance Liquid Chromatography

Detection and Quantification of Benzothiazoles in Exhaled Breath and Exhaled Breath Condensate by Real-time Secondary Electrospray Ionization – High-Resolution Mass Spectrometry and Ultra-High Performance Liquid Chromatography

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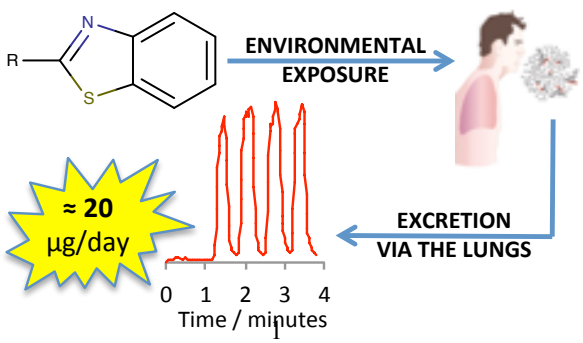
Abstract

2-subtituted benzothiazoles are widely used industrial chemicals whose occurrence in environmental samples has been shown to be ubiquitous. However, knowledge about human exposure to these compounds and their excretion route is still scarce. Here, we demonstrate for the first time the detection of benzothiazole derivatives in exhaled breath. Real-time analysis of breath was carried out by means of secondary electrospray ionization coupled to high-resolution mass spectrometry. This coupling allowed not only the detection of these compounds in breath with a sensitivity in the pptv range but also their robust identification by comparing tandem high-resolution mass spectra from breath and standards. For further confirmation, benzothiazoles were also determined in exhaled breath condensate samples by means of ultra high-performance liquid chromatography. This approach strengthened the identification as a result of excellent matches in retention times and also allowed quantification. An estimated total daily exhalation of ca. 20 µg day^{-1} was calculated for the six benzothiazole derivatives found in breath.

For TOC / Abstract graphic:



Introduction

Benzothiazoles (BTHs) are a class of chemicals that has been used for years in industry, mainly as 2-subtituted derivatives.[1] Their applications are broad, the main one being as vulcanizing agents in rubber production,[2] especially tires.[3] Other applications include their use as biocides in leather, paper, and lumber processing;[4] as corrosion inhibitors in antifreeze fluids;[4] as photosensitizers in photography;[5] and even as food flavors.[6] Due to these widespread applications and their toxicity,[7] BTHs and their transformation products have been an environmental concern since the 80s.[8] Common BTHs such as benzothiazole (H-BTH), 2-aminobenzothiazole (NH_2-BTH), 2-mercaptobenzothiazole (SH-BTH), 2-hydroxybenzothiazole (OH-BTH), 2-methylthiobenzothiazole (SCH_3-BTH), 2-(thiocianomethylthio)benzothiazole (TCMT-BTH), benzothiazole-2-sulfonic acid (BTH-SA), 2-(4-morpholinyl)benzothiazole (Mo-BTH), and N-cyclohexyl-2-benzothiazolamine (NC-BTH) have been extensively studied in aquatic environments[9-15] (rivers, lakes, groundwater, tap, and drinking water, and wastewater treatment plants). These studies have shown that BTHs are ubiquitous at concentrations in the low ng mL^{-1} level. Their presence has also been confirmed in other matrices such as sediments,[8] indoor dust,[16] sewage sludge,[17] or clothing textiles.[18] In spite of all these facts, knowledge about the exposure of human beings to BTHs is very limited.[19] The only biological fluid that has been tested so far is human urine.[15,19] A recent study run in several countries[20] has found urinary concentrations of BTHs corresponding to an estimated daily intake of 4.8 to 18.2 μg/day, however based on the uncertain assumption of a 100% urinary excretion.

Even though exhaled breath has been used for diagnosis since ancient times, it has not received as widespread clinical use as blood and urine have. However, this is being reevaluated[21] and breath is gaining attention as a source of biomarkers not only for noninvasive diagnosis but also for molecular fingerprinting.[22] In this field, our group has shown that secondary electrospray ionization (SESI) is a powerful technique for the real-time analysis of breath[23-25] that, in combination with high-resolution mass spectrometry (HRMS), allows the identification of metabolites found in exhaled breath.[26] Up to date, more than 1000 compounds have been found in exhaled breath[27] including drugs, metabolites and contaminants that, irrespective of their route of administration, have been proven to be excreted, at least partly, via the lungs.[28] Among BTHs, only H-BTH has been detected in breath so far,[29-31] although its occurrence has never been linked with an environmental source.

The aim of this work was to assess the occurrence of BTHs in exhaled breath by means of SESI-HRMS. The quantification of these compounds in breath, something that to the best of our knowledge has not been tried before, should clarify their poorly known excretion route and also eliminate some of the uncertainties concerning the assessment of human exposure. In addition, for better identification of the compounds, exhaled breath condensate (EBC) samples were collected and analyzed by means of ultra-high performance liquid chromatography (UHPLC) coupled to HRMS.

Experimental section

Breath analysis in real time

For real-time breath analysis fifteen subjects (numbered 1-15, Table S1) were asked to breathe through a heated Teflon tube into the inlet of a home-built SESI source (Fig. S1) that was directly flanged to a high-resolution mass spectrometer (TripleTOF 5600+, AB Sciex, Concord, ON, Canada). The exhaled breath was intercepted in the SESI source by a nanoelectrospray plume formed from 0.1% aqueous formic acid. The nanospray voltage was set to +3.6 kV, the declusting potential was set to 20 V, the curtain gas was nitrogen at 2.4 L min^{-1}, and mass spectra from 40 u to 240 u were recorded. The subjects were asked to provide a complete exhalation through a disposable mouthpiece, while keeping the pressure through the sampling tube at 20 mbar (monitored by a manometer), ensuring that each subject breathed at the same flow rate (ca. 2 L min^{-1}). This process was repeated several times per subject and took less than 10 min. For blank samples, room air was introduced into the source by sucking with a pump through the exit tube, restricting the flow at 2 L min^{-1} (Fig. S1). For HRMS/MS analysis, product ion scan experiments were run. The collision energy was set to 30 V with a spread of ±15 V. MS^2 fragmentation pathways were built by means of Sirius 2.[32]

EBC analysis

EBC samples were collected using a home-built device constructed following the recommendations of the ATS/ERS task force[33] (glass cold trap). Ten subjects (numbered 16-25, Table S1) breathed during 10 minutes through their mouths completing deep exhalations. The cooling method was an isopropanol slush bath cooled to -78.5°C with dry ice. The collection time resulted in 1–1.5 mL of EBC (~100 $\mu L \cdot min^{-1}$). After collection, the EBC samples were quickly thawed and transferred to polypropylene vials where they were frozen at -20°C until analysis. For analysis, samples were thawed to 5ºC and transferred to chromatographic vials without any dilution or other sample preparation. 10 µL were then injected into an ACQUITY UPLC system (Waters, Milford, MA, USA). Separation took place in a C18 ACQUITY column (2.1 mm x 100 mm, 1.7 µm, Waters) thermostatized at 25 °C and running a 6-minute gradient of a water/acetonitrile mixture modified with 0.1% formic acid at a flow rate of 0.4 mL min^{-1} (0-4 minutes: from 80/20% to 75/25%, 4-5 minutes: to 30/70% and 5-6 minutes: to 80/20%). For the coupling with an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA), the eluent from the column was introduced into an Ion Max source (+4 kV). Nitrogen was used as sheath, auxiliary and sweep gas at flow rates of 20, 10 and 2 (arbitrary units), respectively. The capillary temperature was set to 275°C. Spectra from 50 u to 250 u were recorded with a resolution of 30,000. Protonated phthalic anhydride (149.02332 u) was used as internal lock, resulting in a mass accuracy below 1 ppm. For HRMS/MS analysis, product ion scan experiments were run with a collision-induced dissociation energy of 37 V. MS^2 fragmentation pathways were built by means of Sirius 2 (University of Jena).[32]

Chemicals

Standards of NH_2-BTH and SH-BTH were obtained from Acros Organics (Geel, Belgium), H-BTH and SCH_3-BTH were obtained from TCI (Eschborn, Germany), OH-BTH was obtained from Maybridge (Geel, Belgium) and Mo-BTH was obtained from Pfaltz and Bauer (Waterbury, CT, USA). Water, acetonitrile and formic acid were of LC-MS quality and were obtained from Sigma-Aldrich (Buchs, Switzerland).

Results and Discussion

Detection of BTHs in exhaled breath by SESI-HRMS

The recent finding of the occurrence of several BTHs in human urine worldwide[20] has shown that human exposure to these compounds may be a global concern. However, the excretion route of BTHs is mostly unknown and there is no reason to disregard other routes apart from urinary excretion. The physico-chemical properties of H-BTH, with a vapor pressure of 0.119 torr (at 25ºC) and a boiling point of 227ºC (at 760 torr), classify this chemical as a volatile organic compound (VOC) and suggest the possibility of some BTHs being also exhaled. In addition, with the exception of BTH-SA, all the compounds studied are not charged at physiological pH (pK_a values for the amino groups in the range 0-4), which should favor the blood to lung partition. Furthermore, it should also be noted that nonvolatile compounds, such may be the case of Mo-BTH (BP: 369ºC), have been found in breath probably arisen by the formation of aerosol droplets from the airway lining fluid[26]. To assess this, exhaled breath samples from fifteen subjects were analyzed as stated in the experimental section. Time traces for six different common BTHs (i.e. H-BTH, NH_2-BTH, SCH_3-BTH, OH-BTH, SH-BTH, and Mo-BTH) are shown in Figure 1. It should be highlighted that all the signals tended to rise at the end of a complete exhalation. This effect has been suggested as a way to discriminate endogenous (i.e. metabolites) vs. exogenous compounds in exhaled breath since, whereas metabolites coming from the alveoli tend to rise, exogenous compounds, which may be present in room air, are gradually diluted by air from the deeper lung and therefore show a trend of falling intensity.[24] In addition, in order to check any possible contamination from plastic ware and lab materials, blank samples consisting of air from the lab were run using the procedure stated in the experimental section. As can be seen in Fig. 1, the background level at the same m/z is insignificant compared to the intensities obtained from exhaled breath, which further confirms the fact that the six BTHs studied are endogenous compounds and not just artifacts from room air.

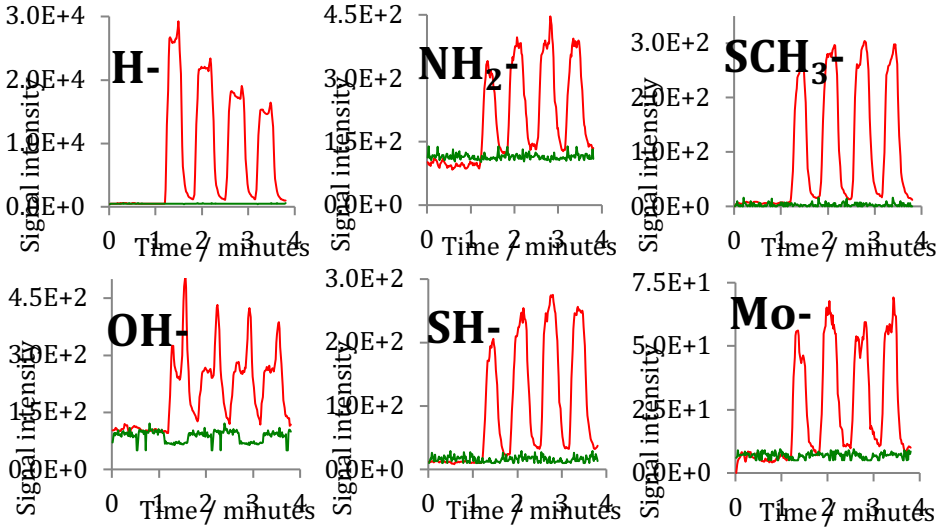


Figure 1. Time traces corresponding to the $[M+H]^+$ adducts of six BTHs (real-time TripleTOF, isolation window: ±10 ppm) extracted from four consecutive exhalations (red, subject #1) and blank samples (green, room air).

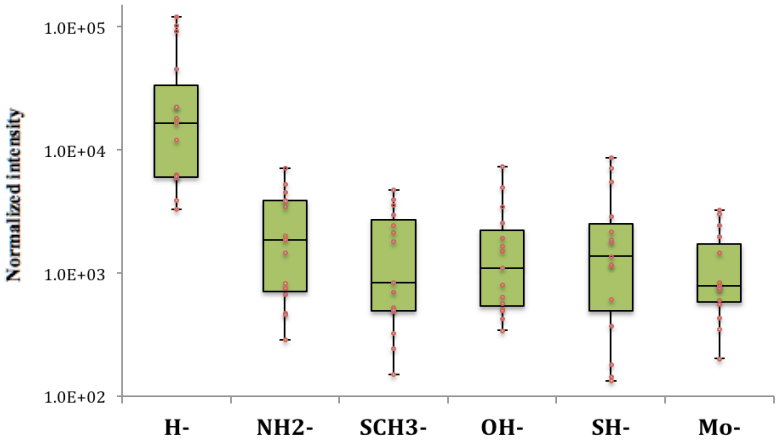


Figure 2. Signal intensities for the six BTHs found in exhaled breath (N=15, signal intensities normalized against total ion intensities).

Figure 2 shows the distribution of intensities among the fifteen subjects for the compounds studied. As can be seen, these six BTHs were detected in all fifteen samples, showing that BTHs can be properly assessed in exhaled breath in real time by means of SESI. Among these compounds, H-BTH was the one showing the highest intensity (Fig. 2), which means that is the main metabolite exhaled. This is in good agreement with the available data regarding urinary excretion,[20] which shows that H-BTH is also the main metabolite excreted via this route. No statistical differences were found (Mann-Whitney U test) for the signal intensities of the six BTHs when comparing between ages or genders (Table S1). However, the comparison between smoking status (smokers vs. non- and ex-smokers) reveals that the signal intensity of SCH_3-BTH is statistically higher in smokers, with a Bonferrini-corrected p-value of 0.009. It should be highlighted that SCH_3-BTH, which is as a byproduct in the leather, pulp, paper and water-treatment industries, has also been listed as a component of tobacco.[34]

Confirmation of the occurrence of BTHs in breath by SESI-HRMS/MS

One of the main disadvantages of breath analysis in real time, e.g., by selected-ion flow-tube mass spectrometry (SIFT-MS), proton-transfer-reaction mass spectrometry (PTR-MS), or SESI is the lack of any separation, which results in overlap of isobaric compounds. This fact clearly limits the identification capabilities of these techniques.[35,36] Over the last years, the coupling of high-resolution mass spectrometers (e.g. SIFT-TOF) has improved the identification step although distinction of compounds with the same elemental composition is not feasible without an additional separation step[36]. To overcome this identification problem, real-time tandem mass spectra can be recorded (i.e. SESI-HRMS/MS) and be compared with standards, yielding a much more robust identification.[26] Therefore, in order to confirm the presence of BTHs in exhaled breath, real-time SESI-HRMS/MS experiments were run and compared with the fragmentation pathways obtained from standards (Figure 3). It should be noted that these fragmentation pathways are highly related, as expected for compounds from the same chemical family, and converge to two main product ions

($C_6H_5^+$ and $C_5H_5^+$, Fig. 3.a) that were found in the tandem mass spectra of all the BTHs studied. These results are in good agreement and expand those previously reported by Reemtsma and coworkers.[1] The comparison of the fragmentation pathways with the spectra obtained from exhaled breath (Fig. 3.b and S2) shows that several peaks from breath match those shown in the pathways. This fact clearly confirms that the peaks in the time traces detected in breath by SESI-HRMS correspond to the target compounds and, therefore, it also confirms the occurrence of these six BTHs in exhaled breath. Other peaks not related to the studied pathways were also found in the tandem mass spectra of breath, probably arising from isobaric compounds in the isolation mass window (Insets in Fig. S2).

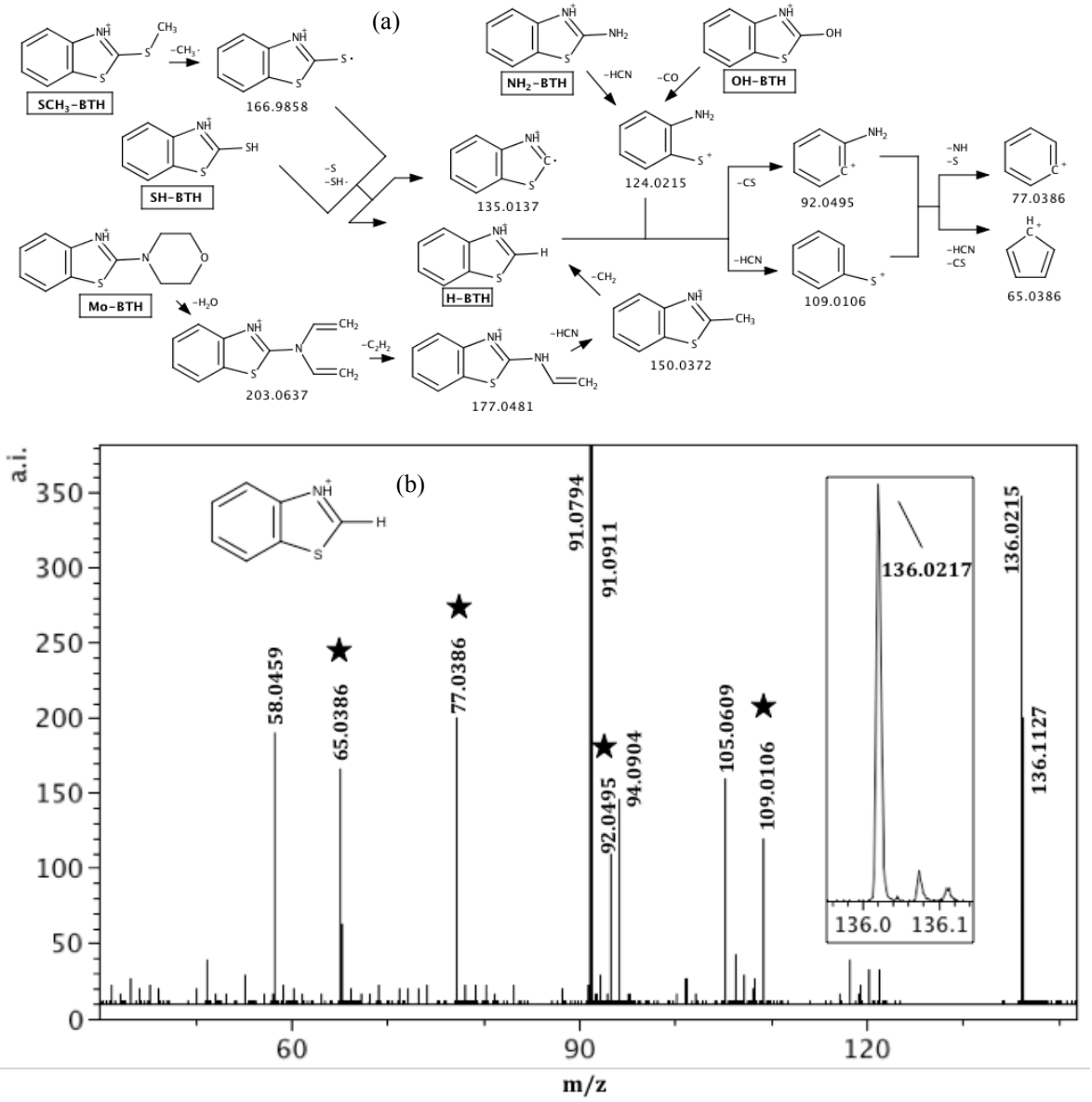


Figure 3. (a) Fragmentation pathways of the six BTHs studied and (b) real-time analysis of exhaled breath: tandem MS spectrum of the compound identified as H-BTH (asterisks mark matches with the corresponding fragmentation pathway).

Determination of BTHs in exhaled breath condensate

Collection of EBC samples has been widely used as a way of analyzing exhaled breath because it allows the use of a chromatographic separation technique prior to MS analysis, which strengthens identification. Therefore, in order to further improve the identification of BTHs in breath, ten EBC samples were collected and analyzed by UHPLC-HRMS in a similar way as has been done before with wastewaters[37], with the exception that no sample treatment was applied. As can be seen in Figure 4, the comparison of retention times from standards and EBC samples agreed very well. Together with the results shown above, we conclude that an unambiguous identification of BTHs in breath has been achieved.

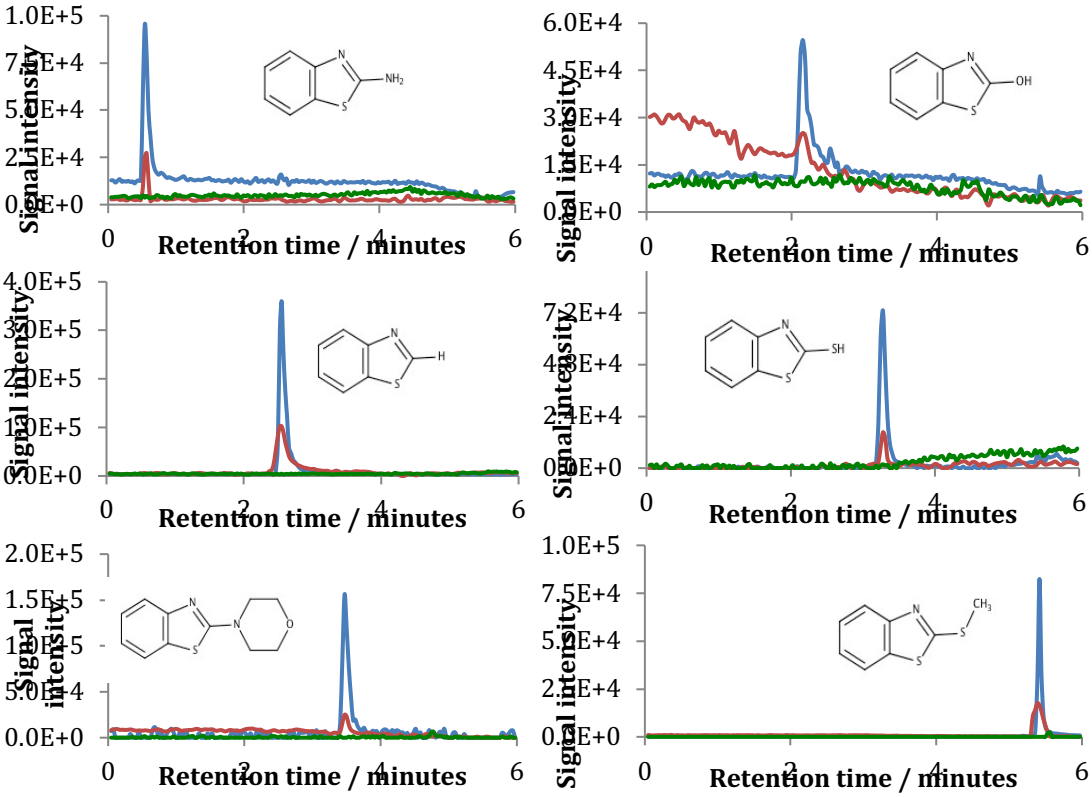


Figure 4. Extracted ion chromatograms of target BTHs from a standard mixture (blue), an EBC sample (red, subject #16) and a blank sample (green).

For quantification, the signal intensities of the EBC samples were blank-corrected and compared with those obtained from standards by means of a calibration curve (LODs: 0.3-5.0 ng mL^{-1}). This approach disregards any matrix effects, but it has been stated before[38] that a diluted matrix such as EBC is virtually free of such effects. The results obtained are shown in Table 1 and Table S2. The geometric mean concentrations in EBC ranged from 6.7 ng mL^{-1} for SH-BTH to 37 ng mL^{-1} for H-BTH. These concentrations were converted to concentrations in exhaled breath by means of the following conversion factors:[39] 1.8±0.5 mL EBC ≙ 119±25 L breath ≙ 15 minutes. The concentrations found in breath were in the range of 10-100 pptv (1-5 ng min^{-1}), which shows the great sensitivity of SESI[24] that matches or surpasses the limits of detection achieved by other techniques such as SIFT-MS[35] and especially PTR-MS.[36,40] Furthermore, this

UHPLC-HRMS approach, even though it cannot be applied in real time, circumvents one of the man disadvantages of real-time SESI, which is that on-line measurements by SESI are currently semi-quantitative at best.

Table 1. Geometric mean concentrations found for the six target BTHs in EBC and breath and estimated daily exhalation

	NH_2-	OH-	H-	SH-	Mo-	SCH_3-
EBC / ppb[1]	11	23	37	6.7	7.0	26
Breath / pptv[2]	27	56	100	15	12	53
Breath / ng min^{-1}	1.3	2.8	4.4	0.8	0.8	3.1
Exhalation / µg day^{-1}	1.8	3.9	6.2	1.1	1.2	4.4

1. ppb: parts per billion (ng mL^{-1}) of EBC
2. pptv: parts per trillion by volume (volume fractions of ideal gases)

It is also possible to calculate the amount of BTHs that are exhaled daily. An estimated total daily exhalation of 19 µg day^{-1} was found for the six BTHs detected in breath. The comparison of this result with that previously found in urine for six BTHs (5-20 µg day^{-1}) [20] clearly suggests that exhalation is at least as important as urinary excretion. Breath concentrations (Table S2) also showed a more homogenous distribution than that previously obtained in urine[20]. It has been suggested that a heterogeneous distribution may reflect different exposure due to differences in personal environment, life styles, etc. Therefore, keeping in mind that environmental differences in this study were limited, a more heterogeneous distribution, similar to that obtained in urine, should be expected for breath studies involving different countries, environments, etc. Anyway, it can be concluded from these results that for a proper study of the excretion of these ubiquitous contaminants and for a proper estimation of the daily intake, exhalation cannot be neglected.

Supporting information available

Characteristics of the studied subjects, scheme and picture of the SESI source, real-time tandem high-resolution tandem mass spectra of exhaled breath, concentrations found for the six target BTHs in EBC and breath, and estimated daily exhalation.

Acknowledgements

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