ORIGINAL RESEARCH

# Analysis of Chemical Simulants in Urine: A Useful Tool for Assessing Emergency Decontamination Efficacy in Human Volunteer Studies

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**Keywords:** benzyl salicylate; chemical incident; emergency decontamination; methyl salicylate; systemic exposure


**Abbreviations:**
BeS: benzyl salicylate
BSTFA: N, O-bis[trimethylsilyl]trifluoroacetamide
EtOAc: ethyl acetate
GC-MS/MS: gas chromatography triple quadrupole mass spectrometry
MeS: methyl salicylate
QC: quality control
SLE: supported liquid extraction
TMCS: trimethylchlorosilane


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## Abstract

**Introduction:** To date, all human studies of mass-casualty decontamination for chemical incidents have relied on the collection and analysis of external samples, including skin and hair, to determine decontamination efficacy. The removal of a simulant contaminant from the surface of the body with the assumption that this translates to reduced systemic exposure and reduced risk of secondary contamination has been the main outcome measure of these studies. Some studies have investigated systemic exposure through urinary levels of simulant metabolites. The data obtained in these studies were confounded by high background concentrations from dietary sources. The unmetabolized simulants have never been analyzed in urine for the purposes of decontamination efficacy assessment.

**Study Objective:** Urinary simulant analysis could obviate the need to collect skin or hair samples during decontamination trials and provide a better estimate of both decontamination efficacy and systemic exposure. The study objective therefore was to determine whether gross skin contamination as part of a decontamination study would yield urine levels of simulants sufficient to evaluate systemic availability free from dietary confounders.

**Methods:** In this study, a gas chromatography-tandem mass spectrometry method was developed for the analysis of two chemical simulants, methyl salicylate (MeS) and benzyl salicylate (BeS), in urine. An extraction and sample clean-up method was validated, enabling quantitation of these simulants in urine. The method was then applied to urine collected over a 24-hour period following simulant application to the skin of volunteers.

**Results:** Both MeS and BeS were present in all urine samples and were significantly increased in all post-application samples. The MeS levels peaked one hour after skin application. The remaining urinary levels were variable, possibly due to additional MeS exposures such as inhalation. In contrast, the urinary excretion pattern for BeS was more typical for urinary excretion curves, increasing clearly above baseline from four hours post-dose and peaking between 12.5 and 21 hours, a pattern consistent with dermal absorption and rapid excretion.

**Conclusion:** The authors propose BeS is a useful simulant for use in decontamination studies and that its measurement in urine can be used to model systemic exposures following skin application and therefore likely health consequences.


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## Introduction

The efficacy of emergency decontamination interventions for persons exposed to a chemical agent are often assessed in human volunteers using chemical simulants.[1-4] Simulants are chemicals that have similar physicochemical properties to agents of concern but that are non-toxic at the doses applied to humans. Only a limited number of simulants for human volunteer trials have been identified for use,[5] the most common of which is methyl salicylate



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(MeS), a colorless, viscous liquid naturally produced by some plants and commonly used as a flavoring in foodstuffs and as a component of topical medications and consumer products. Methyl salicylate has similar physicochemical properties (vapor pressure, water solubility, and biological half-life) to the vesicant chemical warfare agent Sulphur mustard and has therefore seen extensive use in human volunteer decontamination studies where reduced recovery from skin and hair has been used as a measure of efficacy.[1–7]

Benzyl salicylate (BeS) has recently been identified as a simulant for less volatile, more highly persistent threat agents such as dimethyl methylphosphonate and Novichok.[8] Benzyl salicylate is commonly used as an additive in perfumes and cosmetics and has a significantly lower vapor pressure compared to MeS, meaning it persists on skin during longer human decontamination studies and does not readily volatilize like MeS.

Previous human decontamination studies have focused almost exclusively on simulant skin recovery to assess decontamination efficacy. Efficient removal from skin is assumed to reduce systemic exposure. Systemic exposure can be estimated based on skin removal, but measurement in blood or urine would be a more accurate model. Blood collection is invasive, requires skilled phlebotomists to collect, is a complex matrix to analyze, and analyte levels are often lower than in urine. In contrast, urine collection is simple and non-invasive. A previous study has had limited success quantifying metabolites of MeS, such as the salicylic acid glycine conjugate salicyluric acid in urine.[4] High dietary levels of salicylic acid from fruit, vegetables, tea, coffee, and some alcoholic beverages, however, means that urine analysis of salicylic acid and related metabolites such as salicyluric acid as a marker of MeS is problematic, with limited possibility of observing clear differences between levels after decontamination interventions, or even above baseline levels unless either extremely high doses of MeS are applied or draconian dietary and lifestyle restrictions are applied.[4] Benzyl salicylate can be metabolized to salicylic acid, salicyluric acid, and hippuric acid. Hippuric acid is formed from the combination of glycine and benzoic acid, a common metabolic pathway of phenolic compounds which are relatively common in the diet. As such, measurement of BeS metabolites in urine offers little advantage over metabolites of MeS. One option would be to measure the unmetabolized simulants, but there are no reports of direct measurements of MeS or BeS in urine. The sample techniques employed previously for salicylate metabolites have involved sample concentration by evaporation of extracts, which would also remove the volatile parent compounds.[9]

Therefore, the study hypothesis was that gross skin contamination as part of a decontamination study would yield urine levels of MeS and BeS sufficient to evaluate systemic availability with less opportunity for dietary confounders. In this study, the development of a novel method for the detection of MeS and BeS in urine is described, as well as its implementation in a proof-of-principal study in which MeS and BeS was quantified in the urine of six volunteers following application to skin.

## Materials and Methods

### *Study Procedure*

Six participants (three female, three male; age range 22-44 years, median age 27.5) participated in the trial during April 2018. All participants were healthy and were screened before participation. Participants were instructed to avoid foodstuffs suspected to contain MeS and cosmetic products suspected to contain BeS for 24 hours before and after the study during the urine collection period (Supplementary Material; available online only). Ethical approval was independently granted by Public Health England's (London, United Kingdom) Research Ethics and Governance Group (R&D 316).

The study was performed in an enclosed room of volume ~ 70m[3]. All participants were trialed simultaneously. Ten minutes prior to study commencement, participants provided a baseline urine sample. Participants collected urine in a 500ml measuring cylinder, recorded the time and total volume, and a 50ml aliquot was collected.

Simulant application was achieved by pipetting 100μl of each simulant onto the upturned forearm of each participant. The BeS was applied to the right forearm while the MeS was applied to the left forearm. The simulant was applied as a long droplet (~10cm). Participants were instructed to stay as still as possible to avoid simulant run-off until the simulant had dried. Once the simulant was dry, participants could move freely.

Participants remained in the room for 60 minutes. At 60 minutes, the first of eight post-application urine samples were collected using the above protocol. Participants were then permitted to resume their normal daily activities. Over the next 24 hours, urine samples were collected from each participant (at 2-hour, 4-hour, 6-hour, 8-hour, 12.5-hour, 21-hour, and 24-hour post-dose). Urine samples were returned shortly after 24 hours by all participants.

### *Reagents, Materials, and Instrumentation*

Methyl salicylate (methyl 2-hydroxybenzoate, ReagentPlus, ≥99%) and benzyl salicylate (benzyl 2-hydroxybenzoate, 98%) were purchased from Sigma (Gillingham, UK) and 50mg benzyl 2-hydroxybenzoate-3,4,5,6-d4 (BeS-d4, isotopic purity 98%) and 100mg methyl 2-hydroxybenzoate-3,4,5,6-d4 (MeS-d4, isotopic purity 98.6%) were purchased from Qmx Laboratories Ltd. (Thaxted, UK) to be used as internal standards. High-performance liquid chromatography grade (>99.7%) ethyl acetate (EtOAc) was purchased from Fisher Scientific (Loughborough, UK). Derivatizing agent N,O-bis[trimethylsilyl]trifluoroacetamide (BSTFA) plus 1% trimethylchlorosilane (TMCS) was purchased from Thermo Scientific (Hemel Hempstead, UK), and the derivatization catalyst pyridine was purchased from Acros Organics (Thermo Scientific). CP-grade (99.999%) helium used as carrier gas and CP-grade (99.999%) nitrogen used for collision induced dissociation were purchased from BOC (Guildford, UK).

Clear Screw Top Maximum Recovery vials (1.1ml) with 9mm white silicone/cream ptfe septa ultra-low bleed (ULB) screw caps were used for gas chromatography samples. Urine samples were collected in 50ml Gosselin polypropylene graduated self-standing tubes (Fisher Scientific) and were subsampled into 2ml Cryo.S tubes (VWR; Lutterworth, UK).

Gas chromatography triple quadrupole mass spectrometry (GC-MS/MS) analysis was performed using an Agilent 7890B gas chromatograph twinned with an Agilent 7010B triple quadrupole mass spectrometer. Liquid injections (3μl) were achieved using a PAL RTC 120 robotic autosampler.

### *Preparation of Standards*

*Simulant Preparation*—Pure MeS and vegetable oil (100% rapeseed oil; The Co-operative, UK) were mixed in a 1:1 ratio to create the MeS simulant;[10] the BeS simulant was used undiluted.

*Analytical Standards Preparation*—Two separate 1g/ml stock solutions of MeS and BeS in EtOAc were created on ice and



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| Step | Parameter |
|---|---|
| Pre-Treatment | 1. Acidification of 600µl urine with 10µl 15% formic acid.<br>2. Addition of 10µl MeS-d4 and BeS-d4 in $H_2O$ (100ng/ml). |
| Sample Load | 3. Load 350µl Urine.<br>4. Air Push 3s. |
| | 5. Wait 5 minutes. |
| Elution | 6. Add 600µl EtOAc.<br>7. Wait 5 minutes.<br>8. Apply 0.7bar for 30s to elute sample. |

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**Table 1.** Conditions for the Biotage Extrahera Automated Supported Liquid Extraction System used in the Sample Extraction of MeS and BeS from Urine Samples
Note: All extractions were conducted on 96 well plate 400µl sorbent bed ISOLUTE SLE+ cartridges. The system automatically aspirated samples to ensure thorough mixing between pre-treatment and sample load.
Abbreviations: BeS, benzyl salicylate; EtOAc, ethyl acetate; MeS, methyl salicylate.

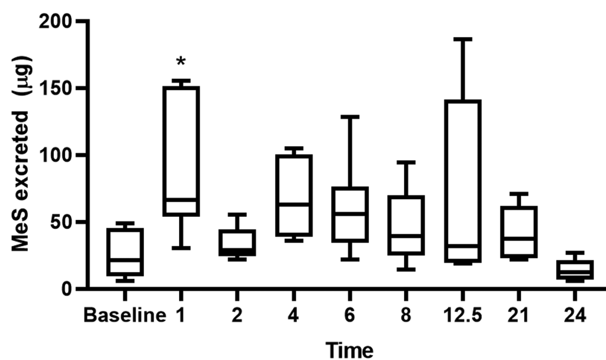

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**Figure 1.** Participants (n = 6) Total Excretion of MeS (µg) Over 24 Hours.
Note: Values given have been normalized against total volume of each urination. 12.5-hour and 22-hour samples are the average values for urine collected before and after bed. Box and whisker plot shows the median and inter-quartile range, together with the maximum and minimum values.
Abbreviation: MeS, methyl salicylate.
* = P <.02 compared to baseline.

diluted to a working solution of concentration 1mg/ml. This was serially diluted with EtOAc to create calibration solutions of concentration 4.6, 46, 230, 460, and 2300ng/ml and three quality control (QC) solutions at 11.5, 115, and 1150ng/ml. A working solution of MeS-d4 and BeS-d4 was created at a concentration of 20µg/ml in EtOAc.

*Sample Spiking of Internal Standard*—For the spiking of urine samples, 20µg/ml standard of MeS-d4 / BeS-d4 was diluted to 100ng/ml in deionized water. Prior to extraction, urine samples were spiked with 10µl of this solution.

*Calibration, QC, and Blank Preparation*—Calibrations, QCs, and blank samples were simply derivatized prior to analysis using a mastermix containing internal standards MeS-d4 and BeS-d4 in a 1µg/ml solution mixed with BSTFA/TMCS and pyridine at a ratio of 10:95:95. The final concentration of internal standard in all calibrations, QCs, and samples was 50ng/ml.

All solutions were stored in sealed headspace vials, in the dark at room temperature, and were cooled on ice before opening.

*Sample Preparation*

Urine was diluted by a factor of 100 with deionized water in preparation for analysis. Supported liquid extraction (SLE; 400µl 96-well plate ISOLUTE SLE+ cartridges; Biotage) of MeS and BeS from urine samples was carried out using an Extrahera Automated Sample Preparation System (Biotage). The extraction method is outlined in Table 1.

Working on ice in a 96-well sample preparation plate, 10µl of 15% formic acid was added to each well to prepare the urine for extraction. Once all wells were acidified, 600µl of urine was added with 10µl of 100ng/ml MeS-d4 and BeS-d4 in deionized water. The plate was capped to reduce evaporative loss during transfer to the sample preparation system. The extraction from 350µl of urine per sample was then carried out according to the parameters in Table 1.

Once extracted, each sample was removed from the collection plate and chemically dried using an excess of magnesium sulphate to ensure no residual water was present prior to derivatization; 20µl of the dehydrated eluents were then derivatized with 10µl of pyridine and 10µl of BSTFA/TMCS at 55°C for 60 minutes prior to analysis.

Instrumentation and method of GC-MS/MS analysis was identical to James, et al[10] with a 3µl injection into gas chromatography rather than a 1µl injection.

**Data Analysis**

All data were analyzed by GraphPad Prism 8.1.2 (GraphPad Software; San Diego, California USA), including medians and interquartile ranges for the box and whisker plots. For comparisons of MeS baseline versus one-hour and two-hour samples, a one-way ANOVA (Tukey's multiple comparisons test) was used.

**Results**

Calibrations showed good linearity with $R^2$ values ranging between 0.9992 and 0.9999 for MeS and 0.9934 and 0.9969 for BeS. All QCs were within tolerable limits for accuracy and precision.[11]

All six participants completed the study with no protocol deviations. All volumes and timings of urine collection were accurately recorded, and all urine samples were returned to the researchers at the correct time. No protocol deviations in terms of foodstuffs or cosmetic product use were recorded from any participant.

All 54 urine samples collected during the study contained detectable levels of MeS and BeS considerably above the assay limits of quantification (4.6ng/ml), with most post-application samples being higher in concentration than the baseline samples. All recoveries are given as total MeS or BeS excreted (µg) per urine sample provided.

For MeS, baseline samples ranged from 5µg to 49µg (concentrations 288.7ng/ml – 300.9ng/ml non-normalized) with the average baseline recovery being 25µg. At 60 minutes, urine showed a significant increase (P <.01) in MeS recovery over baseline for all six participants, followed by a significant decrease (P <.02) in the two-hour sample (Figure 1). The peak excretion of MeS occurred in the 12.5-hour sample with a top excreted amount of 186µg. Cumulative excretion of MeS over 24 hours ranged between 215µg and 689µg (Figure 2).

For BeS, baseline recoveries ranged between 8µg and 34µg (concentrations 257.5ng/ml – 370.3ng/ml non-normalized) with an average recovery of 21µg. The peak excretion of BeS occurred



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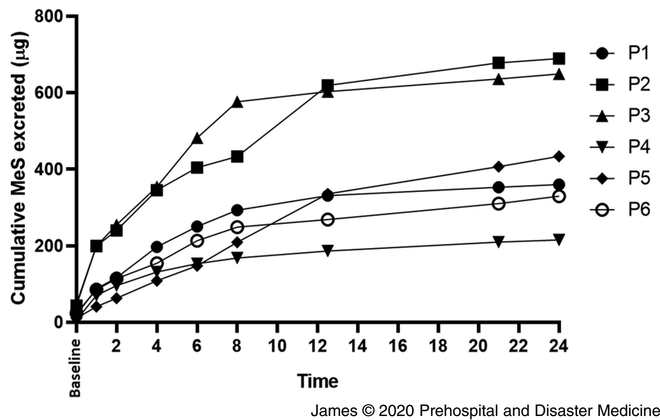


**Figure 2.** Cumulative Excreted Mass of MeS (µg) Over the 24-Hour Period for n = 6 Participants.
Abbreviation: MeS, methyl salicylate.

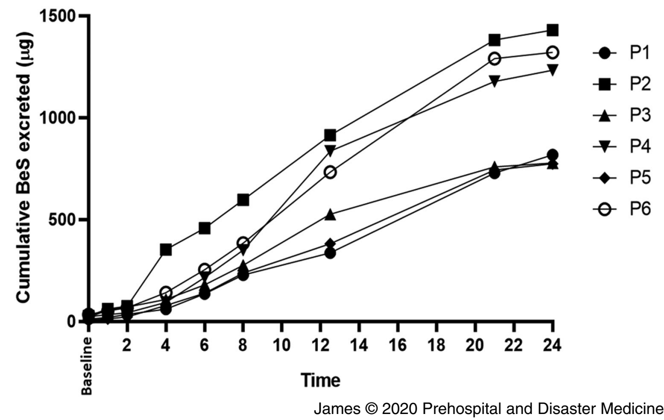


**Figure 4.** Cumulative Excreted Mass of BeS (µg) Over the 24-Hour Period for n = 6 Participants.
Abbreviation: BeS, benzyl salicylate.

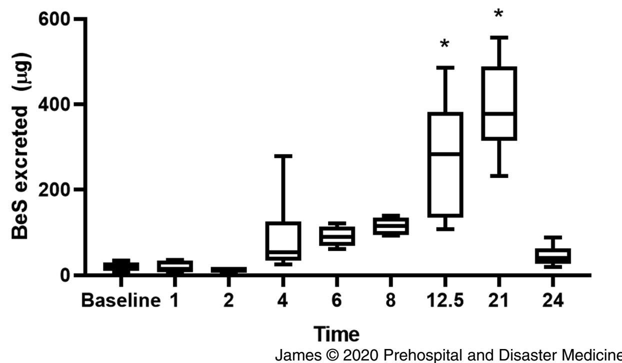


**Figure 3.** Participants (n = 6) Total Excretion of BeS (µg) Over 24 Hours.
Note: Values given have been normalized against total volume of each urination. 12.5-hour and 22-hour samples are the average values for urine collected before and after bed. Box and whisker plot shows the median and inter-quartile range, together with the maximum and minimum values.
Abbreviation: BeS, benzyl salicylate.
* = P <.0001 compared to baseline.

in the 21-hour sample (Figure 3), with the highest value being 557µg. Samples taken at 12.5 hours and 21 hours were both significantly higher than baseline levels (P <.0001). Cumulative excretion of BeS over 24 hours ranged between 773µg and 1430µg (Figure 4).

## Discussion

Analyzing salicylic acid and salicyluric acid can include an evaporative concentration step following liquid-liquid or solid phase extraction. The volatility of MeS, and to a lesser extent BeS, means that this is not an option as these compounds would be lost through evaporation. Here, SLE was employed as it did not involve an evaporative concentration step but was still able to clean up the sample matrix and leave the sample in a suitable solvent for injection directly onto the GC-MS/MS.

Salicylic and salicyluric acid are very common secondary plant metabolites found in human urine as a consequence of dietary intake.[12] Almost all foodstuffs containing salicylates will be in-part metabolized to salicylic acid and further to salicyluric acid. As a result, strict controls on participants' food and drink intake must be observed. However, strict dietary controls on common foodstuffs such as tea, coffee, alcohol, and vegetables could reduce volunteer recruitment and compliance may be poor. Even if dietary restrictions are adhered to, there is the possibility that consumption could still occur from sources not originally known to the researchers. In addition, salicylic acid is a metabolite of aspirin (acetylsalicylic acid). By analyzing parent MeS and BeS rather than the common metabolites they produce, it was hypothesized that the urinary levels post-simulant-application are a result of skin application of simulant rather than foodstuffs or cosmetic items.

Data showed a significant decrease in excreted MeS between the one-hour and two-hour samples. The one-hour peak could potentially be attributed to the fraction of MeS that was inhaled, and thus rapidly bioavailable and excreted. Following this, at four hours, all participants showed another increase in urinary MeS. This could potentially represent the fraction that penetrated though the less-rapid skin absorption route of exposure. For most participants, the levels of MeS generally tailed off after this point, reducing to baseline or below baseline levels by the end of this 24-hour study. Cumulative MeS excretion (Figure 2) showed a reasonable consistency pattern for four participants.

For two participants, higher cumulative levels of MeS were identified throughout the study. These higher levels of excretion could be attributed to an additional, possibly dietary, exposure to MeS, or to reduced metabolism of MeS in these two individuals. It is a recommendation therefore that for contamination studies incorporating MeS, sample size selection may have to take into account this variability in MeS excretion. Further studies are needed to determine additional sources of MeS for participants to avoid. Urine monitoring accompanied by a detailed food diary may identify any factors contributing to urinary MeS.

No significant increase in excreted BeS levels was observed compared to baseline until four hours, when all participants' urinary BeS levels increased (Figure 3). These data suggest that the lower vapor pressure of BeS does not allow for a significant vapor/inhalation contribution to urinary levels as shown by the consistently low BeS levels until four hours. Levels of BeS slowly increased between four hours and 12.5 hours, followed by a large increase overnight and into the post-sleep sample (21 hours) for all participants before dropping to near baseline levels at the 24-hour mark. One participant from the four-hour sample, and two



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additional participants from the 12.5-hour sample, had higher cumulative excretion of BeS resulting in two distinct groups with similar cumulative excretion levels (Figure 4). Like MeS, these observations could be attributed to external factors such as cosmetic products, liquid soaps or fragrances, or due to potential differences in absorption efficiency or metabolism. Once again, this variability between individuals suggests that use of urinary levels to access decontamination studies may require higher numbers of participants to achieve statistical significance than studies that measure simulant remaining on skin.

Both compounds were detectable in urine, with reproducible values across the 24 hours for all participants. The BeS provided more reproducible data than the MeS with participants showing an increase following application and an increasing excretion throughout the day, returning to normal at 24 hours. Levels of BeS were on average approximately twice as high as the levels of excreted MeS, and unlike BeS, total MeS excreted throughout the study did not follow a significant trend, leading to results that are of limited utility.

In addition to the higher dose of BeS applied (MeS was a 1:1 dilution in vegetable oil), the greater urinary BeS recovery could also be attributed to the differences in volatility and lipophilicities between these two compounds, which will contribute to how much of these chemicals cross the skin and enter the circulation. It has previously been demonstrated that following skin application, only 14.7% of the applied dose of MeS simulant (1:1 with vegetable oil) can be recovered after 30 minutes,[10] compared to 28.9% after 60 minutes for the same dose of BeS.[8] The higher persistence of BeS therefore likely results in greater skin penetration than MeS and consequently higher urinary recoveries.

As a marker of systemic exposure to simulant, urinary simulant levels can be used as evidence towards possible decontamination outcomes such as the wash-in-effect. Many studies have assessed whether water-based decontamination strategies increase the dermal penetration of chemicals by changing the properties of the surface of the skin; however to date, all these studies have focused on *in vitro* assessments, and many contradict each other with no discernible conclusion as to whether the wash-in-effect is real or an artefact of experimental procedures. Urinary levels of simulant would be an ideal marker for systemic exposure if applied to controlled *in vivo* wash-in-effect studies.

To the best of the authors' knowledge, this is the first study to demonstrate and quantify the presence of unmetabolized MeS and BeS in human urine, and as such, signifies an important development in the ability to analyze and assess the decontamination efficacy of these chemical simulants in humans. An advantage of targeting parent simulant over their metabolites for quantitation of systemic availability of these salicylates is that this avoids data being compromised by non-targeted exposures from dietary, pharmaceutical, and consumer products that yield salicylate metabolites. Identifying sources of the unmetabolized parent compound is considerably easier than for their metabolites, which are shared with many components of the diet and consumer products. For decontamination studies, urinary quantitation offers a direct measure of systemic availability, so long as other sources of MeS and BeS are avoided. Systemic availability of simulant is better for estimating the likely health improvements from decontamination interventions than levels of simulant remaining on the skin, which better represent the risk of contamination to emergency responders, bystanders, and the wider environment.

## Limitations

While the low recovery of MeS relative to BeS is due to possible evaporation from the skin during the study, it could also be attributed to loss into the headspace of the container following collection of the urine. Participants collected the 50ml aliquot in a container with a watertight - but possibly not airtight - lid. If the container was kept in a warm environment, volatilized MeS may have been lost from the headspace upon opening of the container for urination or sample analysis. While this possibility was minimized by collecting samples in individual containers as opposed to one large container, MeS may still have volatilized, especially in samples of less than 50ml where the headspace would have been larger.

While this study provides essential evidence towards using urine as a marker for systemic exposure to chemical simulants, it is only based on two chemicals. A broader range of chemicals with divergent physicochemical properties, as well has more human volunteers (n = 6 in this study), should be investigated in order to provide more evidence towards systemic availability of chemical simulants to inform decontamination interventions.

## Conclusion

For the first time, both MeS and BeS were detected and quantified in urine samples of human volunteers post-dermal-exposure.

The ability to specifically detect parent simulant (especially BeS) in urine rather than their metabolites is a significant step forward for the determination of decontamination efficacy and could be used to compliment or even replace skin and hair sample analyses.

## Supplementary Material

To view supplementary material for this article, please visit https://doi.org/10.1017/S1049023X20000825

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