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Validation and application of a headspace-gas chromatography-mass spectrometric method for determination of toluene and 2-butanol in urine

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Abstract: Toluene, widely used across various industries, poses significant health risks upon prolonged exposure, such as hallucinations and visual impairment. The Chemical Substances Control Act classifies toluene and butane as hallucinogenic substances, necessitating the strict regulation of their inhalation. Conventionally, toluene exposure has been assessed by measuring hippuric acid levels in urine. However, as hippuric acid is an endogenous substance, its reliability as a marker of toluene inhalation is limited. Similarly, 2-butanol metabolizes to 2-butanone, which is also present in the urine of individuals with certain medical conditions, rendering it unsuitable as a definitive marker of butane abuse. This study aimed to develop and validate a quantitative analysis method for detecting toluene and 2-butanol in urine, addressing challenges associated with assessing exposure to these chemicals. We developed a cryotrap-free quantitative analytical method using an HP-1 column (100 m × 0.25 mm i.d. × 0.5 μm). Sample preparation involved the addition of sodium sulfate and an internal standard to the urine samples. The method demonstrated excellent selectivity, with limits of quantitation and detection at 0.001 and 0.0003 μg/mL for toluene, and 0.01 and 0.002 μg/mL for 2-butanol, respectively. The calibration curve exhibited high linearity ($r^2 > 0.9996$) across a quantitative range (0.002 – 0.2 μg/mL for toluene and 0.4 – 6 μg/mL for 2-butanol), ensuring accurate measurement. This method exhibits significant potential for effectively identifying toluene and butane abuse and offers a valuable tool for regulatory agencies and healthcare professionals to protect public health.

Key words: quantitative analysis, validation, toluene, 2-butanol, urine

1. Introduction

The abuse of volatile organic compounds (VOCs), including toluene and butane, which are found in adhesives, paints, fuels, and butane gas, has emerged as a societal issue that affects individuals across age groups from adolescents to adults, irrespective of social strata or cultures.[1-4] Efforts to mitigate these harmful effects of glue inhalation lead to the creation of glue using cyclohexane as an alternative solvent to toluene. Cyclohexane is not classified as a hallucinogenic substance under the Chemical Substances Control Act. However, toluene and butane, which are VOCs, are commonly abused by teenagers because they are

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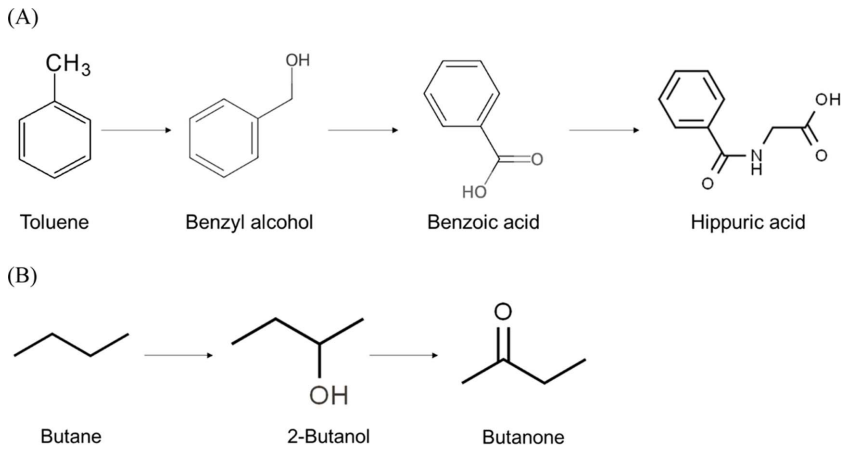

Fig. 1. Metabolic pathway of (A) toluene and (B) butane.

legal, cheap, and readily accessible.[5] Prolonged exposures to these substances can lead to various symptoms, including nausea, hallucinations, and vision problems. Consequently, to control the abuse of toluene and butane, they are designated as hallucinogenic substances under the Chemical Substances Control Act in South Korea.

Toluene is primarily metabolized in the liver, where it is initially oxidized to benzyl alcohol by cytochrome P450 enzymes. The final product, benzoic acid, combines with glycine to form hippuric acid, which is then eliminated through urine (*Fig.* 1).[6,7] Traditionally, measuring hippuric acid concentration has served as a key metabolic indicator for assessing toluene intoxication or exposure levels.[6] However, hippuric acid has several limitations, such as lack of reliability in specific contexts. For instance, hippuric acid can also be produced from other sources, such as the metabolism of dietary components, including benzoic acid found in fruits and food preservatives, which reduces the reliability of the analytical results when hippuric acid is used as the sole marker for toluene exposure. In addition, lifestyle factors, such as smoking and alcohol consumption, can influence hippuric acid levels, leading to erroneous interpretations of exposure levels. Furthermore, under low-exposure environment of toluene, the weak correlation between toluene exposure and urinary hippuric acid makes it a less sensitive indicator than other metabolites, such as *o*-cresol or benzylmercapturic acid, which are considered more reliable markers for monitoring toluene exposure. These limitations have led to an increasing consensus that hippuric acid, which is potentially valuable in specific contexts, is not suitable as a metabolite for measuring toluene exposure, specifically in cases of low-level exposure or when dietary and lifestyle factors are not regulated.[8,9] Direct measurement of toluene provides a more immediate and accurate reflection of recent exposure levels. Unlike metabolic products like hippuric acid or *o*-cresol, which can be influenced by individual metabolic rates or non-occupational sources, direct toluene measurement can clearly indicate acute exposure. Therefore, this study is particularly valuable in situations of acute exposure. In Japan, urinary toluene concentrations exceeding 0.038 μg/mL are used as indicators of exposure.[10]

Since 1995, butane has been regulated as a hallucinogen under the Chemical Substances Control Act in South Korea. Owing to its high volatility, extracting butane directly is challenging, necessitating the use of metabolites as exposure indicators. Butane is metabolized to 2-butanol, which is further metabolized to 2-butanone; however, the consumption of fermented beverages like wine or makgeolli also produces 2-butanone.[11] Moreover, studies have reported its presence in the urine of cancer patients, particularly those with breast or prostate cancer, suggesting its potential to serve as an indicator for cancer diagnosis.[12,13] These

factors limit the use of 2-butanone as a definitive indicator of butane abuse.

Recently, the increased accessibility of regulated drugs via social media has transformed the traditional patterns of abuse. In particular, social media platforms have become key sources for acquiring various drugs, particularly among adolescents and young adults.[14,15] Although this trend persists in certain sectors, ongoing surveillance is necessary. Current research has primarily focused on specific case analyses, such as fatalities resulting from butane inhalation, rather than on establishing comprehensive abuse criteria.[3,16] This finding underscores the persisting risks associated with butane abuse and highlights the need for developing scientific and standardized assessment methods. Concurrent with the ongoing surveillance of butane gas abuse, there exists a critical necessity for research to more precisely identify instances of misuse.

To this end, in this study, toluene and 2-butanol, a butane metabolite, were selected as exposure indicators. The addition of sodium sulfate as a salting-out agent led to the volatilization of previously dissolved toluene and 2-butanol. The addition of sodium sulfate, an electrolyte, causes complete dissociation in water, reducing the solubility of the relatively non-polar toluene and 2-butanol molecules and promoting their volatilization in the upper layer.[17,18] To address the high volatility in biological samples, a headspace gas chromatography-mass spectrometric (HS-GC-MS) method was developed. Although a previous method utilizing a rapid cooling device for HS-GC-MS to concentrate samples and improve separation was developed,[19] maintenance issues led to the adoption of a 100 m column for enhanced separation. The application of this validated method aims to significantly improve the separation of VOCs, facilitate the routine analysis of real samples, and effectively identify cases of substance abuse.

2. Experimental

2.1. Chemicals and reagents

Toluene and 2-butanol were purchased from Sigma Aldrich (St. Louis, MO, USA). Toluene-d_8 and 2-butanone-d_5 were used as internal standards (ISs) and purchased from Sigma Aldrich. Working standard solutions were formulated at 100 μg/mL for toluene and 1000 μg/mL for 2-butanol. The ISs, toluene-d_8 at a concentration of 2 μg/mL, and 2-butanone-d_5 at a concentration of 150 μg/mL, were also prepared as solutions and used as working standard solutions. All standards, solvents, and reagents used were of the highest purity, with water and methanol of liquid chromatography-mass spectrometry grade (where available).

CST Technologies (Great Neck, NY) provided artificial urine, which served as the blank matrix, and Samchun Chemicals (Seoul, Korea) provided sodium sulfate anhydrous, which was used as the salting-out agent.

All calibration curves and quality control (QC) samples were prepared daily by adding standard solutions to the matrix. Matrix effects were evaluated by comparing standard additions to artificial urine and water.

2.2. Instrumental conditions

For the analysis of toluene and 2-butanol, a SHIMADZU HS-20NX headspace sampler was used in combination with a SHIMADZU Nexis GC-2030 and GCMS-AP2020 NX (SHIMADZU Corporation, Kyoto, Japan) to facilitate sample heating and agitation. Analyte separation was achieved using an HP-1 (100 m × 0.25 mm, I.D 0.5 μm) capillary GC column. The GC oven temperature program began at 50 °C for 3 min, then increased to 250 °C at 15 °C/min, and maintained for 8 min. As the carrier gas, helium with high purity was used at a flow rate of 1.1 mL/min and split ratio of 60:1. The complete analysis required approximately 25 min. Characteristic ions for individual samples were identified using a mass spectrometer detector operating in scan mode across a range of m/z 20 – 120.

2.3. Urine samples and sample preparation

Artificial urine served as a blank urine sample to determine the limit of detection (LOD) and selectivity of the method. To assess the ratio of the quantitative

to qualitative ion peak heights for the analytes, blank samples were spiked with toluene and 2-butanol and diluted to 0.01 and 0.2 μg/mL, respectively.

Urine samples (n = 14) from suspected toluene or butane abusers were collected at probation offices for examination. The obtained samples were stored at 4 °C, and those requiring further analysis were separated and kept at -20 °C until further use.

After adding 2 g of sodium sulfate to a headspace-specific test tube, 5 mL of urine sample and 100 μL of IS were added. The tube was quickly capped and mixed for 5 s, with sample injection lasting 0.01 min.

2.4. Method validation

Artificial urine was used to develop the urine analysis method. Validation parameters included selectivity, linearity, LOD, lower limit of quantification (LLOQ), precision, and accuracy. Method selectivity was evaluated by analyzing artificial and drug-free urine to ensure that no interfering reactions between the analytes and IS were observed. Calibration curves were generated using the peak area ratios of the analytes to the IS. Calibration ranges were 0.4 – 6 μg/mL for 2-butanol and 0.002 – 0.2 μg/mL for toluene. The LOD and LLOQ for both compounds were determined after spiking artificial urine with standards. LOD was defined as the analyte concentration with a signal-to-noise ratio (S/N) > 3, whereas LLOQ was the concentration with S/N ≥ 10. In addition, the LLOQ required a coefficient of variation (CV) of less than 20 % for precision and within ± 20 % for bias. Method precision and accuracy were assessed using low-, medium-, and high-concentration QC samples, and six sets of each QC sample were analyzed over three days. Intra- and inter-day precisions (CV) were estimated for the QC samples. Accuracy (bias) was calculated as the percentage difference between the overall mean of the six measurements and nominal concentration. The acceptance criteria for precision were < 20 % CV for LLOQ and < 15 % for other concentrations, whereas the accuracy criteria were within 20 % of the nominal for LLOQ and within 15 % for other concentrations.

3. Results and Discussion

3.1. Salting-out conditions

The efficacy of headspace sampling for volatile compounds depends on the analyte transition from the sample to the gas phase; therefore, varying amounts of sodium (0.5, 1.0, and 2.0 g) were assessed as salting-out agents. *Fig.* 2 illustrates the results for each quantity. Although the impact on toluene was negligible across all tested volumes, the intensity of 2-butanol and 2-butanone-d_5 exhibited a positive correlation with increasing Na_2SO_4 concentration. Quantities exceeding 2.0 g were deemed impractical attributed to the experimental complexity and increased material costs.

3.2. Optimization of instrumental conditions

To increase the reliability of the quantitative analysis method, deuterated IS was used and the analytical method was refined through comparative experiments under diverse conditions. *Table* 1 lists operating parameters of the equipment used in this study.

A previous study utilized a cryogenic oven trapping device coupled with HS-GC-MS and a 30 m column

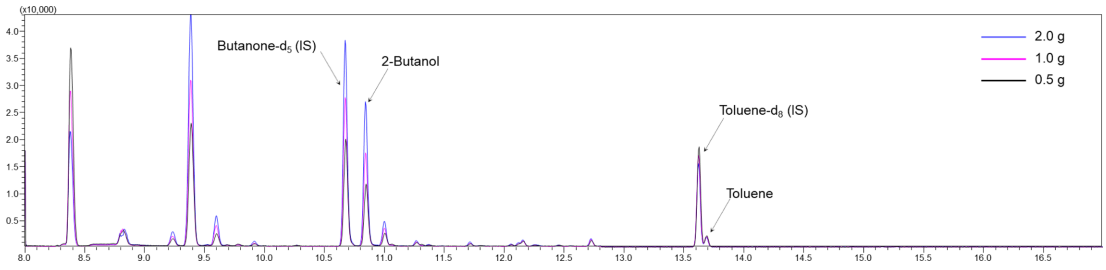

Fig. 2. Salting-out effect of sodium sulfate based on its weight in determining toluene and 2-butanol in urine samples.

Table 1. Operating conditions for HS-GC-MS

Instrumental Condition	Operation Parameters
Headspace Autosampler	
Sample oven temperature	95 °C
Loop temperature	105 °C
Transfer line temperature	120 °C
Thermostat time	10 min
Vial shaking	ON, level 3
Vial pressure	38.4 psi
Pressurization time	2 min
Injection time	0.01 min
Cycle time	35 min
Gas Chromatograph	
Inlet	250 °C (60:1 split ratio)
Helium carrier gas flow rate	1.1 mL/min
Oven temperature program	50 °C for 3 min, then 15 °C/min to 250 °C with a final hold time of 8 min
Mass Spectrometer	
Detect mode	EI, SIM mode
Source temperature	230 °C
Interface temperature	280 °C
Solvent delay	8 min

to enhance the separation capability and sample concentration, resulting in rapid toluene detection at 3.52 min. However, when the trapping device was integrated, maintenance issues, such as repairs, caused problems with routine analysis. To overcome these challenges and improve separation, a 60 m column was initially used. Consequently, the two peaks corresponding to toluene and 2-butanol were not completely separated. Subsequently, a 100 m column was tested, which yielded definitive separation, with toluene eluting at 13.6 min and 2-butanol at 10.8 min.

3.3. Method validation

Artificial urine was used in this study to eliminate the potential butanol detection from fermented beverage consumption. No interference affecting toluene and 2-butanol analysis was observed in either the artificial or human urine samples. The use of artificial urine ensured more consistent experimental conditions, facilitating evaluation of the method's LOD, selectivity, and accuracy. Furthermore, it provides an objective environment for validating the method's performance by eliminating interindividual physiological variations and metabolite concentration fluctuations inherent in real urine samples. *Table* 2 summarizes the retention times and monitored ions for each compound in human urine using HS-GC-MS. LOD and LLOQ values were 0.002 and 0.01 μg/mL for 2-butanol, and 0.0003 and 0.001 μg/mL for toluene, respectively. The intra- and inter-assay precision for both compounds ranged from 0.3 % to 6.1 % CV (%) across all QC samples. Based on six replicate measurements, the accuracy is satisfactory with a bias range of -7.2 % – 6.0 % (*Table* 4). *Fig.* 3 illustrates the representative chromatograms of artificial and human urine samples spiked with standards at the LLOQ levels. The

Table 2. Retention times, molecular weights, and ion monitored for HS-GC-MS of 2-butanol and toluene

Compound	Retention time (min)	Molecular weight	Ions monitored (m/z)		
			Quantifier ion	Qualifier ion	
2-Butanol	10.8	74	45	59	41
Butanone-d_5 (IS)	10.7	77	46	77	-
Toluene	13.6	92	91	92	65
Toluene-d_8 (IS)	13.7	100	98	100	-

Table 3. Method calibration

Analyte	Range (μg/mL)	Slope	y-Intercept	Linearity (r^2)	LOD (μg/mL)	LLOQ[a)] (μg/mL)
2-Butanol	0.4 – 6	2.1161	–0.2228	0.9996	0.002	0.01
Toluene	0.002 – 0.2	0.0271	–0.0010	0.9998	0.0003	0.001

[a)]LLOQ, lower limit of quantitation

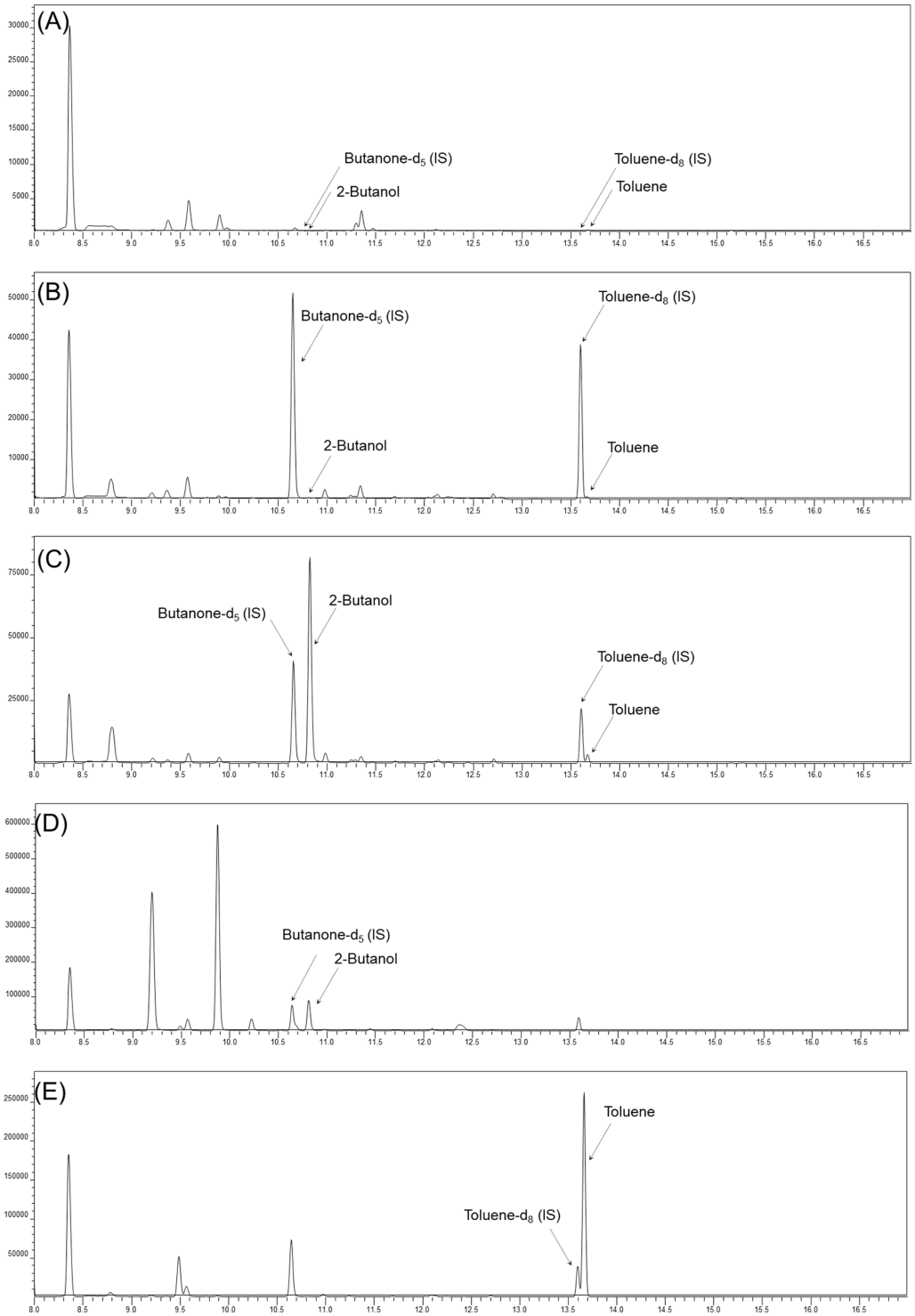

Fig. 3. Representative HS-GC-MS extracted ion chromatograms of toluene and 2-butanol in (A) blank urine without the IS, (B) blank urine with the IS, (C) spiked urine containing LLOQ level, forensic urine sample containing (D) toluene, and (E) 2-butanol.

Table 4. Matrix effects, Intra-day, inter-day precision, accuracy, and stability

Analyte	Nominal concentration (μg/mL)	Matrix effects (%)	Intra-day (n = 6)		Inter-day (n = 18)		Processed stability (%, 12 h)
			Precision (% CV)	Accuracy (% bias)	Precision (% CV)	Accuracy (% bias)	
2-Butanol	0.4	-	1.1	-3.0	1.1	-1.7	100.2
	1.2	93.9	0.7	-7.2	3.1	-5.5	99.0
	2.4	93.0	0.8	-5.1	1.5	-4.4	100.6
	4.8	93.2	0.3	-0.1	1.7	1.3	100.0
Toluene	0.002	-	3.2	5.2	4.7	-0.2	108.3
	0.005	102.5	5.3	6.0	6.1	0.1	103.6
	0.038	100.1	1.8	4.2	3.4	3.9	97.4
	0.150	100.0	2.8	2.6	1.7	3.3	99.1

Table 5. Urinary concentrations of 2-butanol and toluene in abusers

Real sample	2-Butanol (μg/mL)	Toluene (μg/mL)
1	ND[a)]	0.026
2	ND	0.184
3	ND	0.251
4	ND	0.022
5	ND	0.007
6	ND	0.067
7	ND	0.040
8	ND	0.009
9	ND	0.049
10	< LOQ	ND
11	< LOQ	ND
12	< LOQ	ND
13	< LOQ	ND
14	< LOQ	ND
Mean	-	0.073

[a)]ND, not detected

validation results, including the selectivity, calibration range, linearity, LOD, LLOQ, matrix effects, and post-preparation stability of each analyte, are detailed in *Table* 3 and 4. This method exhibited excellent linearity ($r^2 \geq 0.9996$).

3.4. Forensic applications

Butane is metabolized to 2-butanone via 2-butanol; however, the use of 2-butanone as an indicator is limited because it can be detected in urine after consuming fermented beverage, and in urine not exposed to butane. *Table* 5 lists the analysis results of the urine samples (n = 14) from individuals who abused toluene and butane gases. In this study, the results for toluene abusers often exceeded the standard value, indicating that toluene is a relatively common substance of abuse and that its detection can serve as a meaningful indicator in determining abuse. In contrast, 2-butanol was not detected at concentrations above the LLOQ in the analyses. Investigations into the collection process revealed that urine samples suspected of butane abuse were collected approximately 24 h after the estimated time of abuse. These findings underscore the necessity of prompt urine collection to determine butane abuse.

4. Conclusions

This study successfully developed and validated a straightforward and dependable HS-GC-MS method for quantifying toluene and 2-butanol in urine samples, with the aim of detecting toluene and butane abuse. By combining headspace technology with a GC-MS, this method offered simple sample preparation protocols and delivered satisfactory sensitivity for measuring VOCs.

The method's practical applicability was assessed by analyzing real urine samples. Initially, a cryotrap was used for analyte concentration and separation, posing maintenance challenges, such as a breakdown issue. Therefore, a 100 m column was used for analyte separation. The quantity of the salting-out agent, $NaSO_4$, was optimized to facilitate simple sample pretreatment

while maintaining excellent sensitivity.

When applied to urine samples from suspected toluene and butane abusers, prompt urine collection is crucial to accurately determining abuse. These results confirmed toluene's efficacy as a reliable indicator of misuse. However, the critical importance of timely urine sample collection when utilizing 2-butanol as an indicator of butane misuse was emphasized once again.

In conclusion, this study provides valuable insights into the detection of toluene abuse. However, further research is essential to establish definitive criteria for using 2-butanol as a marker of butane exposure. Such criteria would significantly enhance our understanding of butane misuse and its impact on public health, aiding the development of appropriate interventions, and serving as a vital tool in safeguarding public safety.

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