ORIGINAL ARTICLE

Simple and reliable method to simultaneously determine urinary 1- and 2-naphthol using in situ derivatization and gas chromatography-mass spectrometry for biological monitoring of naphthalene exposure in occupational health practice

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Abstract

Objectives: The aim of this study was to develop and validate a simple and reliable gas chromatography-mass spectrometry (GC-MS) method to simultaneously determine urinary 1-naphthol (1-NAP) and 2-naphthol (2-NAP) for biological monitoring of occupational exposure to naphthalene.

Methods: NAPs were derivatized in situ with acetic anhydride after enzymatic hydrolysis, extracted with *n*-hexane, and analyzed using GC-MS. Validation of the proposed method was conducted in accordance with US Food and Drug Administration guidance. A final validation was performed by analyzing a ClinChek®-Control for phenolic compounds.

Results: The linearity of calibration curves was indicated by a high correlation coefficient (>0.999) in the concentration range 1-100 µg/L for each NAP. The limits of detection and quantification for each NAP were 0.30 and 1.00 µg/L, respectively. The recovery was 90.8%-98.1%. The intraday and interday accuracies, expressed as the deviation from the nominal value, were 92.2%-99.9% and 93.4%-99.9%, respectively. The intraday and interday precision, expressed as the relative standard deviation, was 0.3%-3.9% and 0.4%-4.1%, respectively. The ClinChek® values obtained using our method were sufficiently accurate.

Conclusions: The proposed method is simple, reliable, and appropriate for routine analyses, and is useful for biological monitoring of naphthalene exposure in occupational health practice.

KEYWORDS
biological monitoring, gas chromatography-mass spectrometry, naphthalene, naphthol, occupational exposure, urine

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1 | INTRODUCTION

Naphthalene is widely used as a raw material for dye intermediates, synthetic resins, explosives, insect repellents, organic pigments, phthalic anhydride, sterilizing agents, and dyes.[1] Naphthalene has been categorized as a Group 2B (possibly carcinogenic to humans) compound by both the International Agency for Research on Cancer[2] and the Japan Society for Occupational Health (JSOH).[3] It has also been classified as a Category 2 (considered to be carcinogenic for man) compound by the Deutsche Forschungsgemeinschaft (DFG).[4] The American Conference of Governmental Industrial Hygienists (ACGIH) placed naphthalene in the Group A3 (confirmed animal carcinogen with unknown relevance to humans).[5] The occupational exposure limit (OEL) (threshold limit value-time–weighted average, TLV-TWA) for naphthalene proposed by the ACGIH is 10 ppm.[5] OELs have not been proposed by the JSOH or the DFG. More than 30 naphthalene metabolites have been identified in mammals.[6] Among them, 1-naphthol (1-NAP) and 2-naphthol (2-NAP) have been used as biomarkers for exposure to naphthalene in humans.[6] For non-smokers, the DFG has proposed a biological reference value (Biologischer Arbeitsstoff-Referenzwert, BAR) of 35 µg/L (after hydrolysis) for 1-NAP plus 2-NAP in urine.[6] This BAR defines the urinary 1-NAP and 2-NAP level in the population who are not occupationally exposed to naphthalene (ie, the background level). No OEL based on biological monitoring for naphthalene has been proposed by the JSOH; also, no biological exposure index for naphthalene has been proposed by the ACGIH.

In 2015, the Ministry of Health, Labour, and Welfare of Japan designated naphthalene as a chemical substance in the Ordinance on Prevention of Hazards Due to Specified Chemical Substances. Thus, individuals who handle naphthalene in their workplace are required to undergo a medical examination once every 6 months.[1] The medical examination consists of a primary medical examination and, if an occupational physician deems it necessary, a secondary medical examination, at which point the physician may also request urinary 1-NAP and 2-NAP levels to be measured. Therefore, a simple and reliable method for routine analysis of these biomarkers is required by many occupational health-related clinical laboratories.

Several methods to simultaneously determine urinary 1-NAP and 2-NAP have been reported, including gas chromatography-mass spectrometry (GC-MS),[7-11] GC-tandem mass spectrometry (GC-MS/MS),[12-14] GC-high-resolution mass spectrometry (GC-HRMS),[15] high-performance liquid chromatography-fluorescence (HPLC-FL) detection,[16] 3D-HPLC-FL,[17] and high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS).[18] However, these suggested methods are not appropriate for routine analyses. Sample preparation procedures for GC-MS and HPLC-FL methods require complex and time-consuming procedures, such as solid-phase extraction, evaporation, or derivatization. Although the preparation of samples for the 3D-HPLC-FL method is simpler than that required for the GC-MS method, it still requires complex equipment. Furthermore, the equipment required is expensive, so GC-MS/MS, GC-HRMS, and HPLC-MS/MS methods are not often used in most laboratories.

The purpose of this study was to develop and validate a simple and reliable method to simultaneously determine urinary 1-NAP and 2-NAP that was appropriate for routine analysis. In order to achieve this goal, we combined three techniques: a simple derivatization with acetic anhydride, followed by a liquid-liquid extraction, and then a determination using GC-MS.

2 | MATERIALS AND METHODS

2.1 | Materials

1-NAP, 2-NAP, 1-naphthyl acetate (1-NAP-Ac), and 2-naphthyl acetate (2-NAP-Ac) were purchased from Tokyo Chemical Industry. Acetic anhydride and sodium hydroxide solution (0.5 mol/L NaOH, for volumetric analysis) were purchased from FUJIFILM Wako Pure Chemical Corporation. 1-Naphthol-2, 3, 4, 5, 6, 7, 8-d_7 (1-NAP-d_7) was used as an internal standard (IS_1) for method validation and purchased from C/D/N Isotopes Inc. 3-Methyl-1-naphthyl acetate was used as an internal standard (IS_2) for examination of the optimum acetylation reaction conditions and purchased from Tokyo Chemical Industry. Acetonitrile, acetic acid, sodium acetate, and n-hexane were of analytical grade or higher (for high-performance liquid chromatography or for pesticide residue and polychlorinated biphenyl analysis). Ultrapure water was obtained using a PURELAB flex-3 (Organo Corp.). β-Glucuronidase/arylsulfatase from *Helix pomatia* (β-glucuronidase activity, 100 000 Fishman units/mL; acrylsulfatase activity, 800 000 Roy units/mL) was purchased from Roche Diagnostics GmbH. A ClinChek®-Control (Urine Control lyophilized) for phenolic compounds was purchased from RECIPE Chemicals + Instruments GmbH. A mixed standard stock solution of 1-NAP and 2-NAP (each at 1.00 g/L), a mixed standard stock solution of 1-NAP-Ac and 2-NAP-Ac (each at 1.29 g/L), an IS_1 solution (1.00 g/L), and an IS_2 solution (1.39 g/L) were prepared in acetonitrile and stored at 4°C. Urine samples collected from healthy adult volunteers who were non-smokers and were not occupationally exposed to naphthalene were used as blank urine samples. Permission for this study was obtained from the Ethics Committee of the Japan Industrial Safety and Health Association and informed consent was obtained from the volunteers prior to urine sample collection.

2.2 | Instruments

The GC-MS system used was a 7890B gas chromatograph-5977B HES (high-efficiency source) mass spectrometer (Agilent Technologies) equipped with a 30 m × 0.25 mm ID HP-5ms Ultra Inert capillary column with 0.25-μm film thickness (Agilent Technologies). The flow rate of the helium carrier gas was 1.0 mL/min. The injection port and the transfer line temperatures were maintained at 250°C and 300°C, respectively. The temperature of the oven was set to 100°C for 1 minute and then increased to 300°C at a heating rate of 10°C/min. Samples (1 μL) were injected using the pulsed split mode (pulse pressure, 25 psi; pulse time, 1 minute) with a split ratio of 5:1. The mass spectrometer was operated in the electron ionization (EI) mode at an electron energy of 70 eV, an ion source temperature of 280°C, and a quadrupole temperature of 150°C. In order to obtain the mass fragmentation of the derivatives, data were acquired in the selected ion monitoring (SIM)/scan mode with a scan range from m/z 45 to 250. The quantifier ion and the qualifier ion for SIM were m/z 144 and 186 for the acetyl (Ac) derivatives of 1-NAP (1-NAP-Ac) and 2-NAP-Ac, m/z 151 and 122 for IS_1-Ac, and m/z 158 and 200 for IS_2, respectively.

2.3 | Sample preparation

1-NAP and 2-NAP are excreted in the urine as glucuronide and sulfate conjugates.[6] In order to cleave these conjugates, enzymatic hydrolysis was carried out according to the procedure employed in a previous study.[10] IS_1 (5000 μg/L, 20 μL), 0.5 mol/L acetate buffer (pH 5.0, 1 mL), and β-glucuronidase/arylsulfatase (20 μL) were added to a glass test tube containing urine (2 mL). The tube was vortexed and then incubated for at least 16 hours at 37°C. The sample was allowed to cool at room temperature and then the acetylation reaction was performed by the addition of 0.5 mol/L NaOH solution (1 mL) and acetic anhydride (50 μL), with immediate vortexing for 10 seconds. After the tube was left to stand for 10 minutes, n-hexane (1 mL) was added and the mixture was gently shaken for 2 minutes, taking care not to form an emulsion. The sample was centrifuged at 1870× g for 10 minutes. A 1 μL aliquot of the extraction solution layer (upper layer) was injected into the GC-MS system. In the optimization of the acetylation reaction conditions, IS_2 (13 900 μg/L, 20 μL) was added after acetylation instead of IS_1.

2.4 | Validation of the method

Validation of the method was conducted in accordance with the US Food and Drug Administration (FDA) guidance.[19] To obtain a calibration curve, the mixed standard solutions of NAPs were added to blank urine samples at seven concentrations, ie, 1, 5, 10, 25, 50, 75, and 100 μg/L. These samples were prepared in triplicate and analyzed using the aforementioned procedure. Calibration curves were constructed by plotting the peak area ratio of Ac derivatives of each NAP to the Ac derivative of IS_1 vs their respective concentrations. The reproducibility of the method we developed, defined as precision, was evaluated by analyzing the urine samples containing three concentrations (1, 25, and 100 μg/L) of each NAP on the same day (five replicates; intraday reproducibility) and over 3 consecutive days (five replicates; interday reproducibility). Accuracy and precision were expressed as the deviation from the nominal value and as the relative standard deviation, respectively. Recovery was calculated by comparing the peak area ratio of Ac derivatives of each NAP to the Ac derivative of IS_1 in spiked urine samples with those of standard solutions. The limit of detection (LOD) and limit of quantification (LOQ) were defined as the quantities of each NAP in urine that corresponded to three and ten times the baseline noise, respectively. The final validation was performed by comparing the analytical values of ClinChek® obtained using the proposed method with the certified values of ClinChek®.

3 | RESULTS

3.1 | Optimization of acetylation reaction conditions

The influence of the volume of acetic anhydride was examined using urine samples containing 100 μg/L of each NAP to determine the optimal acetylation reaction conditions. When the volume of acetic anhydride ranged from 10 to 200 μL, the yields of 1-NAP-Ac at 20, 50, 100, and 200 μL were 96.7 ± 4.0%, 99.6 ± 1.1%, 99.3 ± 0.9%, and 97.2 ± 0.6%, respectively, and those of 2-NAP-Ac at 20, 50, 100, and 200 μL were 100.4 ± 4.4%, 102.5 ± 0.8%, 102.6 ± 1.4%, and 101.2 ± 0.4%, respectively. In contrast, each NAP Ac-derivative was not almost detected at 10 μL.

3.2 | Validation

Typical EI mass spectra and structures of each NAP Ac-derivative are presented in Figure 1. The molecular ions ($M^{+•}$) of each NAP Ac-derivative were observed at m/z 186 for 1-NAP-Ac and 2-NAP-Ac and m/z 193 for 1-NAP-d_7-Ac. The base peaks were observed at m/z 144 for 1-NAP-Ac and 2-NAP-Ac and m/z 151 for 1-NAP-d_7-Ac. These corresponded to $[C_{10}H_7OH]^+$ for m/z 144 and $[C_{10}D_7OH]^+$ for m/z 151. Other major fragment ions
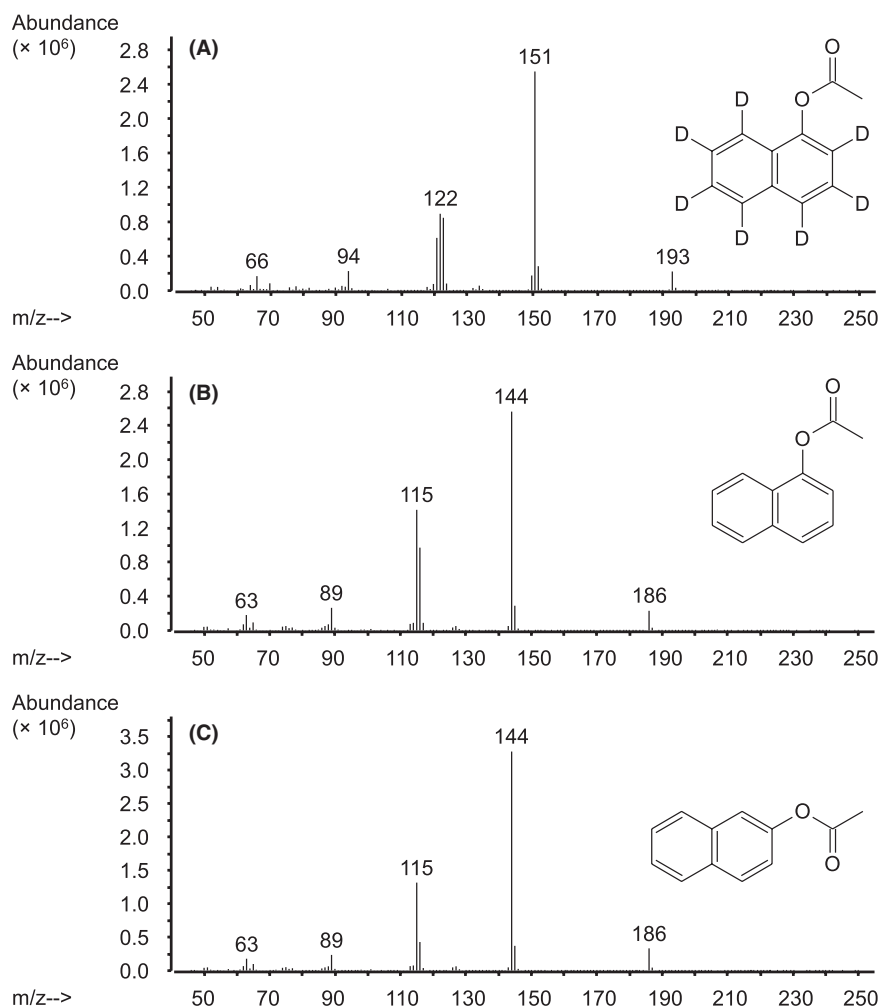
FIGURE 1 Typical electron ionization mass spectra and structures of the acetyl derivatives of (A) 1-naphthol-d_7 (1-NAP-d_7-Ac, internal standard), (B) 1-naphthol (1-NAP-Ac), and (C) 2-naphthol (2-NAP-Ac)

were observed at m/z 115 ($[C_9H_7]^+$ ion) for 1-NAP-Ac and 2-NAP-Ac as well as m/z 122 ($[C_9D_7]^+$ ion) for 1-NAP-d_7-Ac. These mass spectra were used to assign each NAP Ac-derivative peak on the chromatograms, and each of the base peaks was chosen as a quantifier ion. The m/z 115 and m/z 193 were not used as qualifier ions because of the high background noise due to column bleeding. Figure 2 presents typical mass chromatograms for standard spiked urine, ClinChek® Level II, and blank urine. The peaks of Ac derivatives of NAPs were sharp and symmetrical and revealed an absence of disturbances caused by endogenous interference.

The linearity of the calibration curves was indicated by a high correlation coefficient (>0.999) in the concentration range 1-100 µg/L for each NAP. The LOD and LOQ for each NAP was 0.30 and 1.00 µg/L, respectively. The recovery was 90.8%-98.1%. Intraday and interday accuracies were 92.2%-99.9% and 93.4%-99.9%, respectively. Intraday and interday precisions were 0.3%-3.9% and 0.4%-4.1%, respectively (Table 1). Table 2 shows the analytical values of ClinChek® obtained using the proposed method along with the certified values of ClinChek®.

4 | DISCUSSION

The objective of this study was to develop and validate a simple and reliable method for the simultaneous determination of urinary 1-NAP and 2-NAP using GC-MS that is appropriate for routine analysis. Various GC methods have been reported to simultaneously determine urinary 1-NAP and 2-NAP.[7-15] A crucial point for accurate determination using a GC method is derivatization to transform NAPs into less polar compounds in order to obtain better chromatographic behavior. Therefore, N-($tert$-butyldimethylsilyl)-N-methyltrifluoroacetamide,[7,12] acetic anhydride,[8,9] N, O-bis (trimethylsilyl) trifluoroacetamide,[10] pentafluorobenzyl bromide,[11] trifluoroacetic anhydride,[13] 1-chloro-3-iodopropane,[14] or N-methyl-N-(trimethylsilyl)-trifluoroacetamide[15] has been used as derivatization reagents. However, most of these reagents are inappropriate for routine analyses because they require dehydration by an evaporation procedure prior to derivatization. This evaporation procedure is time-consuming and can potentially result in the loss of the target compound. Derivatization utilizing an acetylation reaction with acetic anhydride to produce naphthyl acetate can be carried out in

FIGURE 2   Typical reconstructed mass chromatograms for (A) standard spiked urine (25 µg/L for each); (B) ClinChek® Level II (RECIPE Chemicals + Instruments GmbH) (1-NAP, 20.7 µg/L; 2-NAP, 17.1 µg/L); and (C) blank urine. The peaks of the acetyl derivatives of (1) 1-naphthol-d_7 (1-NAP-d_7-Ac, internal standard), (2) 1-naphthol (1-NAP-Ac), and (3) 2-naphthol (2-NAP-Ac) are indicated
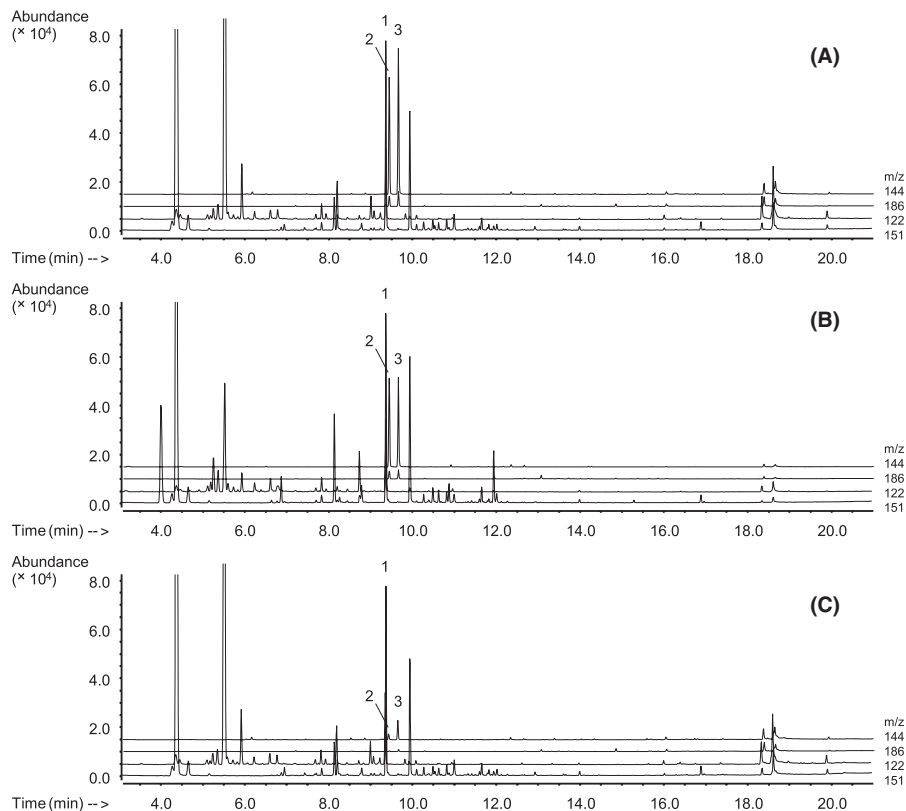

TABLE 1   Intra- and interday coefficients of variation of the proposed method

Spiked urine concentration (µg/L)	Recovery (n = 5)		Intraday (n = 5)[a]			Interday (n = 15)[b]		
	Mean ± SD (%)	RSD (%)	Mean ± SD (µg/L)	RSD (%)	Accuracy (%)	Mean ± SD (µg/L)	RSD (%)	Accuracy (%)
1-NAP								
1	90.8 ± 1.1	1.2	0.92 ± 0.01	1.2	92.2	0.93 ± 0.03	3.1	93.4
25	94.8 ± 0.9	0.9	24.34 ± 0.22	0.9	97.4	24.24 ± 0.20	0.8	97.0
100	93.5 ± 0.3	0.3	99.70 ± 0.29	0.3	99.7	99.84 ± 0.43	0.4	99.8
2-NAP								
1	92.7 ± 3.7	3.9	0.95 ± 0.04	3.9	94.6	0.95 ± 0.04	4.1	94.7
25	98.1 ± 1.1	1.1	23.93 ± 0.26	1.1	95.7	23.87 ± 0.18	0.8	95.5
100	97.1 ± 0.5	0.5	99.87 ± 0.54	0.5	99.9	99.85 ± 0.61	0.6	99.9

Abbreviation: RSD, relative standard deviation.

[a]Intraday reproducibility analysis was performed on a single day.

[b]Interday reproducibility analysis was performed over three consecutive days in five replicates.

an aqueous solution without needing an evaporation step.[8,9] Moreover, the naphthyl acetate produced can be efficiently extracted using a hydrophobic organic solvent because it is a hydrophobic compound. Therefore, we adopted acetic anhydride as the derivatization reagent and developed a rapid and simple sample preparation procedure.

A crucial determinant of successful acetylation is the formation of naphtholate anions in solution, since these anions react with acetic anhydride in aqueous solution.[20] The

optimal pH for this reaction occurs within a range high enough to increase the amount of naphtholate anions and low enough to avoid the destruction of acetic anhydride before the reaction is complete.[20] In previous studies, potassium carbonate solution[8] and sodium tetraborate solution[9] were used as buffer solutions. Potassium carbonate solution should be used with caution because it generates carbon dioxide, which can induce a large increase in pressure inside the reaction vial. Mattarozzi et al[9] used sodium tetraborate solution (pH

TABLE 2 Results for urinary naphthols in ClinChek (levels I and II)[a]

| Metabolites | Level I | | | Level II | | |
| | This method (n = 5) | Certified | | This method (n = 5) | Certified | |
	Mean ± SD	Mean	Confidence intervals	Mean ± SD	Mean	Confidence intervals
1-NAP	5.04 ± 0.03	5.37	3.76-6.98	21.0 ± 0.6	22.0	17.6-26.4
2-NAP	5.57 ± 0.08	5.72	4.00-7.44	17.3 ± 0.4	18.8	15.0-22.6

[a]Concentrations in μg/L.

9.2) to avoid this problem, however, there was insufficient formation of naphtholate anions because the naphtholate anion is formed at a higher pH than the pK_a value of naphthol (1-NAP, 9.34; 2-NAP, 9.51).[21,22] We utilized a 0.5 mol/L NaOH solution as a base solution and adjusted the pH of the sample solution to 12-13. Moreover, we optimized the acetylation reaction conditions. The naphtholate anions in the sample solution immediately reacted with acetic anhydride, and vortexing for 10 seconds was enough to ensure that the reaction was completed. After vortexing, the sample was allowed to settle for 10 minutes to ensure hydrolysis of any excess acetic anhydride. The acetylation yield of each NAP approached 100% after the addition of 20 μL acetic anhydride, and these yields were sustained up to the addition of 200 μL of acetic anhydride. In contrast, each NAP Ac-derivative was not almost detected after the addition of 10 μL acetic anhydride. This discrepancy is likely due to the pH of the sample solution after acetylation. Following the acetylation reaction, the pH of the sample solution gradually decreased, depending on the hydrolysis of acetic anhydride. The approximate pH values of the sample solutions after acetylation with 10, 20, and 50 μL of acetic anhydride were 12, 6, and 5, respectively. Hence, our data suggested that at least 20 μL of acetic anhydride was required to stabilize the NAP-Acs generated in the sample solution because the high pH of the solution may induce decomposition of the NAP-Acs. Therefore, we selected an acetic anhydride volume of 50 μL to ensure the stability of the NAP-Acs generated.

The proposed method was validated in accordance with the US FDA guidance and met their specified criteria (Table 1). The results of a ClinChek® analysis also revealed that the proposed method was sufficiently accurate because our mean ± SD values fell within the certified confidence intervals (Table 2).

The LOD and LOQ of our method were 0.30 and 1.00 μg/L, respectively. The LODs of previous methods were 0.1-0.27 μg/L for GC-MS,[8,11] 0.06-0.3 μg/L for GC-MS/MS,[12-14] 0.012-0.018 μg/L for GC-HRMS,[15] 10 nmol/L (approximately 1.4 μg/L) for HPLC-FL,[16] and 0.5-1.5 μg/L for 3D-HPLC-FL.[17] The LOQs of previous methods were 0.1-3.8 μg/L for GC-MS,[7,9,10] 0.2-0.9 μg/L for GC-MS/MS,[12,13] and 0.001-0.01 μg/L for HPLC-MS/MS.[18] The GC-HRMS[15]

and HPLC-MS/MS[18] methods are much more sensitive than our method. However, the instruments used in these methods are not only expensive but the GC-HRMS[15] method requires an evaporation procedure prior to derivatization, while the HPLC-MS/MS[18] method requires solid-phase extraction followed by a concentration procedure. The HPLC-FL[16,17] methods do not require a derivatization procedure and their LODs are similar to those of the present method, but they do require a sample cleaning procedure to avoid interference from other substances. The method developed by Sams[16] requires a solid-phase extraction procedure and has a relatively narrow linear range. The method described by Preuss et al[17] requires a column-switching system. Therefore, these methods are unsuitable for routine analysis used in the biological monitoring of occupational exposure. Although the sensitivity of our method was similar or lower than the sensitivity of the previous methods, it was sensitive enough for measuring 1/10 of the NAP BAR value. Therefore, the proposed method will be useful in occupational health practice.

In conclusion, a simple and reliable GC-MS method was developed and validated to simultaneously determine urinary 1-NAP and 2-NAP. The proposed method is appropriate for routine analyses and will be useful for biological monitoring of naphthalene exposure in occupational health practice.

DISCLOSURE
Approval of the research protocol: The protocol of this study was approved by the Ethics Committee of the Japan Industrial Safety and Health Association. *Informed consent*: Informed consent was obtained from the volunteers. *Registry and the registration no. of the study/trial*: N/A *Animal studies*: N/A *Conflict of interest*: N/A.

AUTHOR CONTRIBUTIONS
AT conceived the idea and drafted the manuscript. AT, SN, and ON collected the data. AT, AN, and SY analyzed the

data. SN and TK provided technical expertise. HO and SO provided theoretical expertise. YE, HM, and GE provided critical feedback and contributed to the preparation of the manuscript.

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