Ethenesulfonyl Fluoride as a New Derivatization Reagent of Water-Soluble Substances for the GC/MS Analysis in Forensic Science

白木, 亮輔

Doctoral Thesis

Ethenesulfonyl Fluoride as a New Derivatization Reagent of Water-Soluble Substances for the GC/MS Analysis in Forensic Science

Ryosuke Shiraki

Graduate School of Systems Life Sciences

Kyushu University

2024

Contents

Abbreviation

BSTFA	*N,O*-bis(trimethylsilyl)trifluoroacetamide
ECD	electron capture detector
EI	electron ionization
ESF	ethenesulfonyl fluoride
eV	electron volt
EWG	electron withdrawing group
GC	gas chromatography
GC/MS	gas chromatography-mass spectrometry
HFBA	heptafluorobutyric anhydride
HS-GC/MS	headspace gas chromatography/mass spectrometry
i.d.	inside diameter
IC	ion chromatography
IS	internal standard
LC	liquid chromatography
LC/MS	liquid chromatography-mass spectrometry
m-	meta
Me	methyl
MeOH	methanol
min	minute(s)
MS	mass spectrometry
MSTFA	*N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide
n-	normal
NICI	negative ion chemical ionization

NMR	nuclear magnetic resonance
Nu	nucleophile
o-	ortho
OPA	*ortho*-phthalaldehyde
p-	para
PFB	pentafluorobenzyl
PFBBr	pentafluorobenzyl bromide
RSD	relative standard deviation
S/N	signal-to-noise
SD	standard deviation
sec	second(s)
SIM	selective ion monitoring
SuFEx	sulfur (VI) fluoride exchange
SWGDRUG	Scientific Working Group for the Analysis of Seized Drugs
TBB	tribromobenzene
TDMBA	tetradecyldimethylbenzylammonium
TFAA	trifluoroacetic anhydride
TFAI	trifluoroacetyl imidazole
TMS	trimethylsilyl
TMSIM	trimethylsilylimidazole

CHAPTER 1

Introduction

1.1 Forensic Science

Forensic science applies natural sciences such as physics, chemistry, and biology to analyze physical evidence to solve and prove crimes and accidents. In Japan, public institutions such as the National Research Institute of Police Science and forensic science laboratories are involved in criminal investigations. They research the latest technologies and methods for analyzing physical evidence collected during crime and accident investigations. Recently, with the increasing complexity and sophistication of crimes and accidents, the importance of objective evidence has increased. Forensic science encompasses several specialized fields, including forensic chemistry, forensic medicine, forensic biology, forensic engineering, forensic documents, forensic psychology, forensic geology, and forensic botany. In forensic chemistry, applications of chemistry and pharmacology are used to analyze controlled substances, pharmaceuticals, toxic substances (such as pesticides, toxic gases, heavy metals, natural toxins, chemical weapons, etc.), household products, organic solvents, oils, fibers, and more.

1.2 Mass Spectrometry

In the field of forensic chemistry, a wide range of chemical substances is dealt with, including abused drugs (methamphetamine, narcotics, marijuana, etc.), pharmaceuticals (sedatives, tranquilizers, antidepressants, antiepileptics, etc.), pesticides (organophosphates, phosphoramidates, etc.), inorganic poisons (cyanide, azide, etc.), organic solvents (alcohols, cresol, thinners, etc.), toxic gases (hydrogen sulfide, ammonia, carbon monoxide, etc.), chemical weapons (sarin, tear gas, etc.), and household products (detergents, bleaches, etc.). Actual samples vary widely, ranging from biological specimens such as blood, urine, organs, stomach contents, and hair, to food, plants, soil, and more. In forensic chemistry, it is essential to accurately identify trace amounts of target substances from samples with complex matrices and limited quantities.

Various analytical instruments are used to identify these substances. Among these, mass spectrometry (MS) is a particularly important technique. MS is a highly reliable method of substance identification based on mass spectra, which reflect chemical structures. As a result, it is a widely used analytical method in forensic chemistry. The results of forensic sample analyses are critical evidence in court proceedings, and the effectiveness of these identified forensic samples has been recognized in court proceedings.[1-3] According to guidelines by the Scientific Working Group for the Analysis of Seized Drugs (SWGDRUG), analytical methods are classified based on their identification capabilities (Table 1.1). SWGDRUG stipulates that substance identification should be performed using at least two analytical methods based on different principles. It is recommended to combine one method from the highest identification capability category A with at least one method from categories A to C.[1] In addition, MS is widely used as a confirmatory method in various fields, including clinical trials,[4] doping testing,[5]

food analysis,[6,7] and environmental analysis.[8]

Table 1.1 Categories of analytical techniques.[1]

Category	Analytical techniques
A (Selectivity through structural information)	Infrared spectroscopy
	Mass spectrometry
	NMR
	Raman spectroscopy
	X-ray diffractometry
B (Selectivity through chemical and physical characteristics)	Capillary electrophoresis
	Gas chromatography
	Ion mobility spectrometry
	Liquid chromatography
	Microcrystalline tests
	Supercritical fluid chromatography
	Thin layer chromatography
	Ultraviolet/visible spectroscopy
C (Selectivity through general or class information)	Color tests
	Fluorescence spectroscopy
	Immunoassay
	Melting point
	Pharmaceutical identifiers

MS can be combined with various separation techniques. Gas chromatography (GC) and liquid chromatography (LC) are commonly used. Gas chromatography-mass spectrometry (GC/MS) is suitable for identifying low-polarity, volatile, and thermally stable chemicals. In GC/MS, chemicals are dissolved in organic solvents and injected into the instrument for analysis. GC/MS provides characteristic electron ionization (EI) mass spectra that reflect the structure of chemical substances, greatly aiding in the identification

of unknown samples. On the other hand, liquid chromatography-mass spectrometry (LC/MS) is more compatible with high-polarity, non-volatile, and thermolabile chemicals. Although each MS method has its strengths and weaknesses, they enable the analysis and identification of a wide range of chemicals.

1.3 Derivatization

1.3.1 The Purpose of Derivatization in Chromatography

Forensic samples often have complex matrices that require various preprocessing steps before introduction to analytical instruments. However, chemicals with similar structures, such as positional isomers, cannot be separated by preprocessing alone, and their separation by chromatography is also challenging. In addition, the resulting EI mass spectra are often similar, making identification difficult and requiring optimization of separation conditions and various parameter studies for each chemical.

One of the preprocessing methods in chromatography is derivatization, a technique long used in GC. The purpose of derivatization by GC is as follows: (1) improving separation, (2) increasing detection sensitivity, (3) gaining insight into chemical structure by tracking changes in separation behavior, and (4) deriving insight into chemical structure from changes in detector response.[9,10] (1) In derivatization, increased volatility and stability can facilitate separation. It can also convert optical isomers to diastereomers, allowing separation without chiral columns. (2) By converting substances into compounds that are more easily detected by detectors such as electron capture detectors, flame photometric detectors, thermal ionization detectors, and mass spectrometers, increased sensitivity in detection can be achieved. (3) If specific components in a sample can be derivatized, comparing the separation behavior before and after derivatization can help distinguish these specific components. (4) Changes in selective detector responses and retention times before and after derivatization can sometimes indicate the amount and type of functional groups.

1.3.2 The Purpose of Derivatization in MS

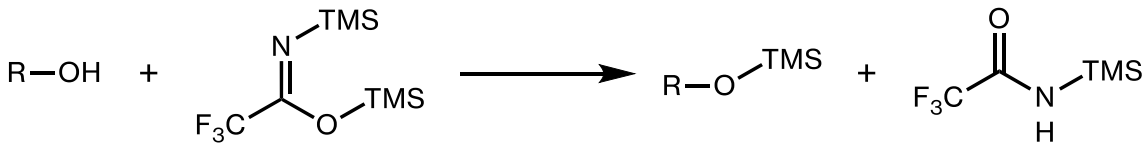
In addition to the purposes of derivatization in chromatography, derivatization is performed in MS for several other reasons. In the analysis of abused drugs such as stimulants and narcotics, which often have similar chemical structures, the resulting EI mass spectra are often similar. Therefore, it is common practice to introduce strong electron trapping groups in negative ion chemical ionization (NICI) to facilitate the analysis of compound structures, such as double bond positions and substituent types, and to increase detection sensitivity.[9]

In addition, toxic gases such as hydrogen sulfide and ammonia, which are highly volatile, are typically analyzed using headspace gas chromatography/mass spectrometry (HS-GC/MS). HS-GC/MS requires a liquid sample to be placed in a glass vial, sealed, and heated to transfer the analyte. This gas phase is then introduced into the instrument for analysis. However, the EI mass spectra obtained by this method often lack specificity due to the small and simple molecular weight of the analytes.[11] Therefore, by introducing compounds into the functional groups of the analyte and converting the gas samples into stable compounds, it becomes possible to obtain characteristic fragments that reflect the chemical structure of the analytes.[10,12]

1.3.3 Types of Derivatization

The method of derivatization varies depending on the functional groups of the chemicals being analyzed. Common derivatization methods include silylation,[13-15] acylation,[16-18] and esterification.[19,20]

Silylation is used for compounds with hydroxyl, carboxyl, and amino groups (Fig. 1.1). Reagents for trimethylsilyl (TMS) derivatization include *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), trimethylsilylimidazole (TMSIM), and *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA). However, during silylation, both the silylating agents and the silylated compounds can be degraded by water in the system. Therefore, it is important to be aware of water contamination, not only in the reaction solution but also in the experimental apparatus. In addition, silylated compounds can decompose in the presence of moisture in the air, so it is important to avoid humidity.[9]



N, O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA)

Fig. 1.1 TMS derivatization of alcohols with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA).

Acylation is used for compounds containing amino and hydroxyl groups (Fig. 1.2). Acylation reagents include trifluoroacetic anhydride (TFAA), trifluoroacetyl imidazole (TFAI), and heptafluorobutyric anhydride (HFBA). The advantage of this reagent is that it is capable of incorporating halogens into the compound, thus improving the response in the electron capture detector (ECD).[9]

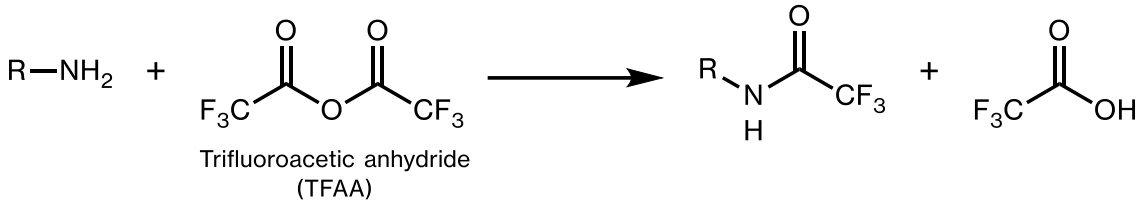


Fig. 1.2 Trifluoroacetyl derivatization of amines with trifluoroacetic anhydride (TFAA).

Esterification is an effective technique for compounds with carboxyl groups, such as fatty acids and organic acids (Fig. 1.3). Carboxylic compounds can have high polarity and sometimes low volatility. This can cause problems in GC analysis where these compounds may not elute properly or may cause tailing. Diazomethane is commonly used as an esterification reagent. However, diazomethane is known for its hazardous properties, including carcinogenicity and explosiveness. To mitigate these drawbacks, methods using trimethylsilyldiazomethane, an improved variant of diazomethane, have been developed.[9]

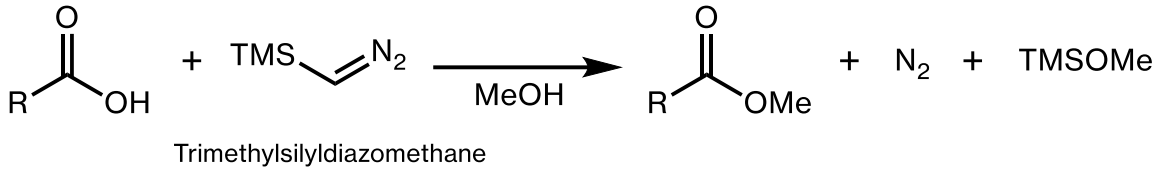


Fig. 1.3 Methyl esterification of fatty acids with trimethylsilyldiazomethane.

In addition, derivatization with pentafluorobenzyl bromide (PFBBr) is commonly used (Fig. 1.4). This reagent is less reactive with water molecules and can therefore be used to derivatize water-soluble nucleophilic substances. As a result, it is used in forensic science to derivatize several important analytes, such as cyanide ion,[21] sulfide ion,[22,23] carboxylic acid,[24] azide ion,[25] and phenol,[26] among others.[27] However, PFBBr has disadvantages, as the reagent has tearing properties that make it difficult to handle, and it has been noted that it lacks reproducibility.[28,29]

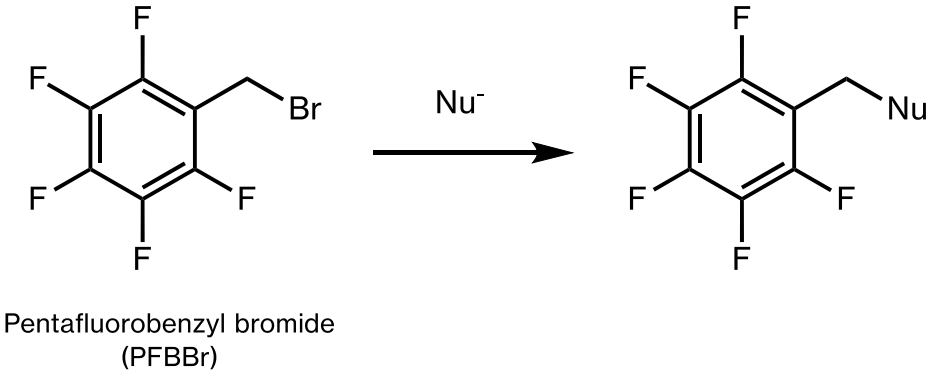


Fig. 1.4 Derivatization reactions of nucleophiles with pentafluorobenzyl bromide (PFBBr).

1.4 Ethenesulfonyl Fluoride

Ethenesulfonyl fluoride (ESF) has been reported to be an important compound in click chemistry, which refers to a branch of chemistry that utilizes click reactions.[30-32] Click reactions are bond-forming reactions that are effective in molecular modification and allow the synthesis of target compounds without the need for extensive optimization of reaction conditions. As the term "click" suggests, these reactions can quickly and conveniently link two molecules together, similar to the snapping of a seat belt buckle. Key reaction groups in click chemistry include (1) addition-cyclization of unsaturated bonds, (2) ring-opening reactions of strained electrophilic heterocycles, (3) non-aldol type carbonyl reactions, and (4) additions to carbon-carbon multiple bonds.[30]

Among these, the Huisgen reaction between azide and alkyne groups is particularly important (Fig. 1.5). This reaction proceeds on heating at high temperatures, but the addition of a monovalent copper catalyst can accelerate the reaction rate and allow it to proceed under milder conditions.[33]

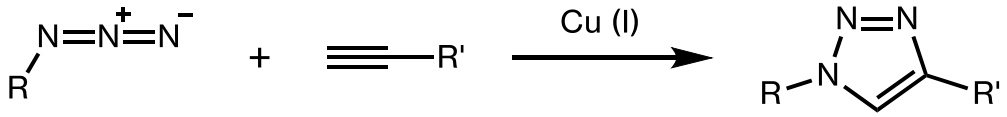


Fig. 1.5 Huisgen reaction.

In recent years, sulfur (VI) fluoride exchange (SuFEx) reactions have been proposed as a new form of click chemistry involving reactions with sulfur (VI) fluorides (Fig. 1.6).[34-36] In these reactions, sulfonyl fluorides (R-SO_2F) react with nucleophiles (Nu) under appropriate conditions to yield sulfonyl compounds (R-SO_2Nu). Notably, sulfonyl fluorides are highly stable compounds, resistant to heat and reduction, and possess the intriguing ability to react rapidly with nucleophiles even under aqueous conditions.[37]

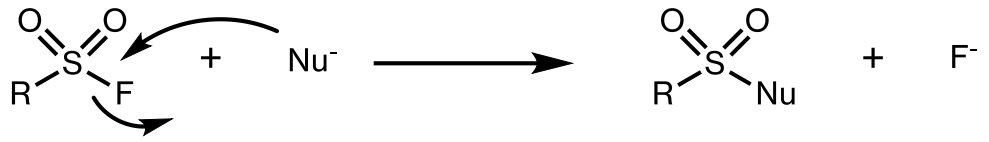


Fig. 1.6 Reactions using sulfur (VI) fluoride exchange (SuFEx).

On the other hand, a similar substituent to sulfonyl fluoride is sulfonyl chloride, but the stability of sulfonyl chloride is not as high. This can be understood from the bond energies. The bond energy of S (VI)-F is 90.5 ± 4.3 kcal/mol, while that of S (IV)-Cl is only 46 ± 4 kcal/mol. Therefore, sulfonyl chloride does not undergo homolytic cleavage like sulfonyl fluorides.[38] The use of this specific reactivity under certain conditions allows the reaction to occur only in certain environments. The synthesis of sulfonyl fluorides requires the use of sulfuryl fluoride (SO_2F_2).[39,40] However, SO_2F_2 is toxic and difficult to handle, so it is recommended to work in a fume hood.[41] On the other hand, the use of ESF allows the convenient introduction of the sulfonyl fluoride group.[35,42] ESF is a compound in which a vinyl group is directly attached to a sulfonyl fluoride group (Fig. 1.7).

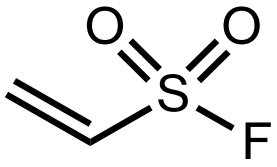


Fig. 1.7 Structure of ethenesulfonyl fluoride (ESF).

This compound has been utilized in various fields, including dyes,[43] functional materials such as ion-exchange membranes,[44] and in the medical sector.[45,46] ESF, with its electron deficiency at the *α,β*-unsaturated bond, is a highly reactive Michael acceptor.[35,43] Consequently, it can react with *N*-, *S*-, *O*-, and *C*-nucleophiles (Fig. 1.8). On the other hand, it is reported that ESF does not significantly react with compounds containing hydroxyl groups, such as water and aliphatic alcohols.[37,47-50]

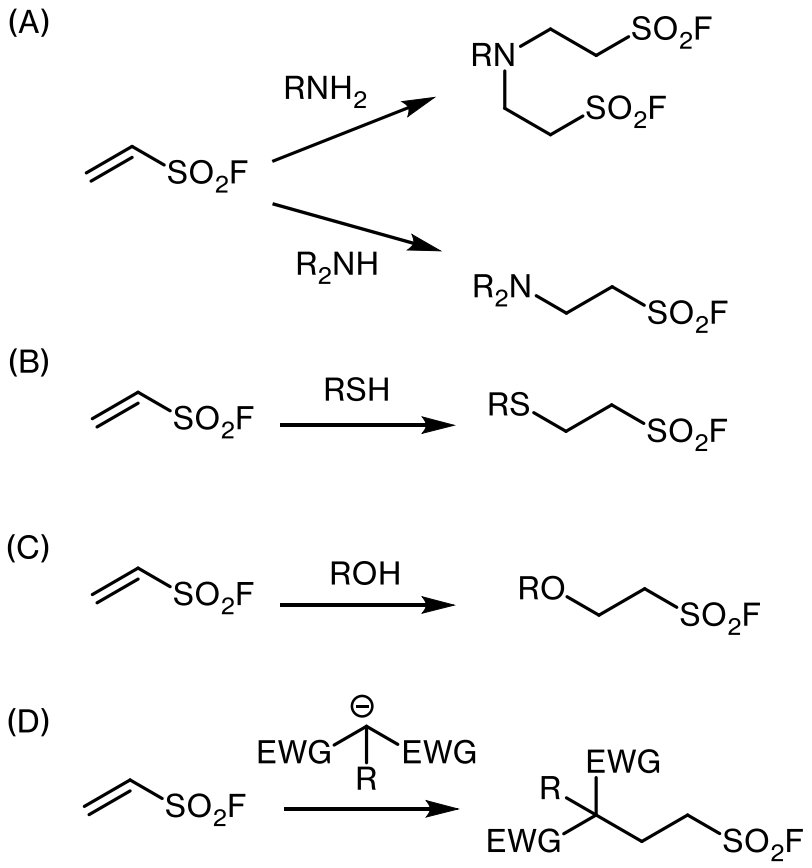


Fig. 1.8 Reaction of ESF with nucleophiles: reaction with *N*-nucleophiles (A), *S*-nucleophiles (B), *O*-nucleophiles (C), and *C*-nucleophiles (D).

By using ESF as a Michael acceptor and initiating Michael addition reactions with various nucleophiles, it becomes possible to conveniently introduce sulfonyl fluoride groups, which are reaction sites in SuFEx reactions. Therefore, ESF is a very important compound in the field of click chemistry.

1.5 Purpose of This Study

In this study, we selected ammonia, sulfide ion, and cresol from among the chemical substances that are difficult to analyze with GC/MS and that are frequently encountered in numerous crimes and accidents. We developed a rapid, simple, and highly specific GC/MS analytical method using ESF, a derivatizing reagent that can react in water.

1.6 Overview of This Thesis

This thesis consists of five chapters. The summary is as follows:

In Chapter 2, we developed an analytical method for ammonia in aqueous solutions. Ammonia was found to react with ESF in water to form a stable compound, N-ESF_3. The optimal conditions for the derivatization reaction were investigated in terms of reaction temperature, reaction time, pH, and extraction solvent. The stability of the resulting N-ESF_3 was also evaluated, as well as its quantitative analysis by GC/MS. Simulated samples containing different types of interfering substances were used to test the applicability of the method. In addition, commercially available products containing ammonia were analyzed and compared with conventional methods to assess the reliability of this new approach.

In Chapter 3, we developed an analytical method for sulfide ion in whole blood. Hydrogen sulfide (H_2S) in blood exists as sulfide ion and can form stable S-ESF_2 by reaction with ESF. The optimal conditions for derivatization were investigated in terms of protein precipitation solvent, reaction time, pH, and extraction solvent. The stability of the resulting S-ESF_2 was also evaluated, and it was possible to perform quantitative

analysis with good reproducibility using GC/MS. Furthermore, the reliability of this method was investigated in comparison with conventional methods.

In Chapter 4, we developed a method to identify isomers of cresol in whole blood. The phenolic hydroxyl group in cresol reacts with ESF to form a stable cresol-ESF compound. This compound allowed easy separation of cresol isomers by GC/MS. The protein precipitation solvent, reaction time, pH, and extraction solvent were investigated to determine the optimal conditions for derivatization. The stability of the resulting cresol-ESF was also evaluated, and a quantitative analysis was carried out.

Finally, Chapter 5 provides a comprehensive summary of the research findings and conclusions of this study.

1.7 References

1. Scientific Working Group for the Analysis of Seized Drugs (SWGDRUG), Quality Assurance/Validation of Analytical Methods, available from https://www.swgdrug.org/approved.htm, accessed Apr 20, 2023.
2. Society of Forensic Toxicologists Inc. / American Academy of Forensic Sciences, SOFT / AAFS Forensic Laboratory Guidelines, available from http://www.the-ltg.org/data/uploads/guidelines/soft-guidelines_2006.pdf, accessed May 1, 2023.
3. Cooper Gail A. A., Paterson Sue, Osselton M. David, The United Kingdom and Ireland Association of Forensic Toxicologists. *Science & Justice*, **50**, 166-176, 2010.
4. European Medicines Agency (EMA), ICH M10 on bioanalytical method validation - Scientific guideline, available from https://www.ema.europa.eu/en/documents/scientific-guideline/ich-guideline-m10-bioanalytical-method-validation-step-5_en.pdf, accessed Jul 15, 2022.
5. World anti-doping agency (WADA), International Standard for Testing and Investigations, available from https://www.wada-ama.org/en/testing-and-investigations, accessed Jul 27, 2023.
6. Di Stefano V., Avellone G., Bongiorno D., Cunsolo V., Muccilli V., Sforza S., Dossena A., Drahos L., Vekey K., Applications of liquid chromatography-mass spectrometry for food analysis. *J. Chromatogr. A*, **1259**, 74-85, 2012.
7. Cao G., Li K., Guo J., Lu M., Hong Y., Cai Z., Mass Spectrometry for Analysis of Changes during Food Storage and Processing. *J. Agric. Food Chem.*, **68**, 6956-6966, 2020.

8. Ribeiro C., Ribeiro A. R., Maia A. S., Goncalves V. M., Tiritan M. E., New trends in sample preparation techniques for environmental analysis. *Crit. Rev. Anal. Chem.*, **44**, 142-185, 2014.

9. Blau K., Halket J., Nakamura H., Handbook of derivatives for chromatography, 2nd ed. pp 281-308, *Wiley*, 1996.

10. Stanczuk A., Morris N., Gardner E. A., Kavanagh P., Identification of (2-aminopropyl)benzofuran (APB) phenyl ring positional isomers in internet purchased products. *Drug Test. Anal.*, **5**, 270-276, 2013.

11. McAdam K. G., Tetteh J., Bishop L., Digard H., Cote J., Lubbe S., Liu C., A Combined Study of Headspace Volatiles using Human Sensory, Mass Spectrometry and Chemometrics. *Sci. Rep.*, **10**, 7773, 2020.

12. Kranenburg Ruben F., Verduin Joshka, Stuyver Laura I., de Ridder Renee, van Beek Annique, Colmsee Erik, van Asten Arian C., Benefits of derivatization in GC–MS-based identification of new psychoactive substances. *Forensic Chem.*, **20**, 2020.

13. Sobolevsky Tim G., Revelsky Alexander I., Miller Barbara, Oriedo Vincent, Chernetsova Elena S., Revelsky Igor A., Comparison of silylation and esterification/acylation procedures in GC-MS analysis of amino acids. *J. Sep. Sci.*, **26**, 1474-1478, 2003.

14. Nishio T., Toukairin Y., Hoshi T., Arai T., Nogami M., Determination of 3-chloro-l-tyrosine as a novel indicator of chlorine poisoning utilizing gas chromatography-mass spectrometric analysis. *Leg. Med.*, **47**, 101782, 2020.

15. Elie M. P., Baron M. G., Birkett J. W., Injection port silylation of gamma-hydroxybutyrate and trans-hydroxycrotonic acid: conditions optimisation and characterisation of the di-tert-butyldimethylsilyl derivatives by GC-MS, *Analyst*, **137**, 255-262, 2012.

16. Zampolli M., Meunier D., Sternberg R., Raulin F., Szopa C., Pietrogrande M. C., Dondi F., GC-MS analysis of amino acid enantiomers as their N(O,S)-perfluoroacyl perfluoroalkyl esters: application to space analysis. *Chirality*, **18**, 279-295, 2006.

17. Kusch P., Knupp G., Hergarten M., Kozupa M., Majchrzak M., Identification of corrosion inhibiting long-chain primary alkyl amines by gas chromatography and gas chromatography–mass spectrometry. *Int. J. Mass Spectrom.*, **263**, 45-53, 2007.

18. Awad T., DeRuiter J., Clark C. R., GC-MS Analysis of Acylated Derivatives of The Side Chain and Ring Regioisomers of Methylenedioxymethamphetamine, *J. Chromatogr. Sci.*, **43**, 296-303, 2005.

19. Stoffel W., Chu F., Ahrens E. H., Analysis of Long-Chain Fatty Acids by Gas-Liquid Chromatography. *Anal. Chem.*, **31**, 307-308, 1959.

20. Ecker J., Scherer M., Schmitz G., Liebisch G., A rapid GC-MS method for quantification of positional and geometric isomers of fatty acid methyl esters, *J. Chromattogr. B*, **897**, 98-104, 2012.

21. Kage S., Nagata T., Kudo K., Determination of cyanide and thiocyanate in blood by gas chromatography and gas chromatography-mass spectrometry. *J. Chromatogr. B*, **675**, 27-32, 1996.

22. Kage S., Nagata, T., Kimura, K., Kudo, K., Extractive alkylation and gas chromatographic analysis of sulfide. *J. Forensic Sci.*, **33**, 217-222, 1988.

23. Kage S., Ikeda H., Ikeda N., Tsujita A., Kudo K., Fatal hydrogen sulfide poisoning at a dye works. *Leg. Med.*, **6**, 182-186, 2004.

24. Kikuchi M., Sato M., Ito T., Honda M., Application of a new analytical method using gas chromatography and gas chromatography-mass spectrometry for the azide ion to human blood and urine samples of an actual case. *J. Chromatogr. B*, **752**, 149-157, 2001.

25. Rosenfeld J., Mureika-Russell M., Yeroushalmi S., Solid-supported reagents for the simultaneous extraction and derivatization of carboxylic acids from aqueous matrices: Studies of optimization of reaction conditions, *J. Chromatogr. A*, **358**, 137-146, 1986.

26. Cline R. E., Todd G. D., Ashley D. L., Grainger J., McCraw J. M., Alley C. C., Hill R. H., Gas Chromatographic and Spectral Properties of Penta-fluorobenzyl Derivatives of 2,4-Dichlorophenoxyacetic Acid and Phenolic Pesticides and Metabolites, *J. Chromatogr. Sci.*, **28**, 167-172, 1990.

27. Kouichiiro T., Yasuo S., Simultaneous Detection of Anionic Toxic Substances Using GC/MS Analysis of Pentafluorobenzyl Derivatives. *Japanese J. Sci. Technol. Identif.*, **7**, 19-35, 2002.

28. Varlet V., Giuliani N., Palmiere C., Maujean G., Augsburger M., Hydrogen sulfide measurement by headspace-gas chromatography-mass spectrometry (HS-GC-MS): application to gaseous samples and gas dissolved in muscle. *J. Anal. Toxicol.*, **39**, 52-57, 2015.

29. Iwamuro Y., Murakami T., Ishimaru R., Sakamoto Y., Chinaka Satoshi, Determination of hydrogen sulfide in whole blood by headspace-gas chromatography/mass spectrometry. *Jpn. J. Forens. Sci. Technol.*, **25**, 151-158, 2020.

30. Kolb H. C., Finn M. G., Sharpless K. B., Click Chemistry: Diverse Chemical Function from a Few Good Reactions. *Angew. Chem., Int. Edit.*, **40**, 2004-2021, 2001.

31. Evans R. A., The Rise of AzideAlkyne 1,3-Dipolar Click Cycloaddition and its Application to Polymer Science and Surface Modification. *Aust. J. Chem.*, **60**, 384-395, 2007.

32. Dong J., Krasnova L., Finn M. G., Sharpless K. B., Sulfur(VI) fluoride exchange (SuFEx): another good reaction for click chemistry. *Angew. Chem., Int. Edit.*, **53**, 9430-9448, 2014.

33. Tornøe Christian W., Christensen Caspar, Meldal Morten, Peptidotriazoles on Solid Phase: [1,2,3]-Triazoles by Regiospecific Copper(I)-Catalyzed 1,3-Dipolar Cycloadditions of Terminal Alkynes to Azides. *J. Org. Chem.*, **67**, 3057-3064, 2002.

34. Dong J., Sharpless K. B., Kwisnek L., Oakdale J. S., Fokin V. V., SuFEx-based synthesis of polysulfates. *Angew. Chem., Int. Edit.*, **53**, 9466-9470, 2014.

35. Meng Y. P., Wang S. M., Fang W. Y., Xie Z. Z., Leng J., Alsulami. Hamed and Qin. Hua-Li, Ethenesulfonyl Fluoride (ESF) and Its Derivatives in SuFEx Click Chemistry and More. *Synthesis*, **52**, 673-687, 2019.

36. Barrow A. S., Smedley C. J., Zheng Q., Li S., Dong J., Moses J. E., The growing applications of SuFEx click chemistry. *Chem. Soc. Rev.*, **48**, 4731-4758, 2019.

37. Chen Q., Mayer P., Mayr H., Ethenesulfonyl Fluoride: The Most Perfect Michael Acceptor Ever Found? *Angew. Chem., Int. Edit.*, **55**, 12664-12667, 2016.

38. Kiang T., Zare R. N., Stepwise bond dissociation energies for the removal of fluorine from thionyl fluoride and sulphuryl fluoride. *J. Chem. Soc., Chem. Commun.*, 1228-1229, 1980.

39. Narayan S., Muldoon J., Finn M. G., Fokin V. V., Kolb H. C., Sharpless K. B., "On water": unique reactivity of organic compounds in aqueous suspension. *Angew. Chem., Int. Edit.*, **44**, 3275-3279, 2005.

40. Lekkala R., Moku B., Rakesh K. P., Qin H. Li., Applications of sulfuryl fluoride (SO_2F_2) in chemical transformations, *Org. Chem. Front.*, **6**, 3490-3516, 2019.

41. Schneir A., Clark R. F., Kene M., Betten D., Systemic fluoride poisoning and death from inhalational exposure to sulfuryl fluoride. *Clin. Toxicol. (Phila.)*, **46**, 850-854, 2008.

42. Liu L., Yang Y., Xi R., Zhang X., Xu W., Liu X., Li Y., Liu P., Wang Z., Global Wet‐Reduced Nitrogen Deposition Derived From Combining Satellite Measurements With Output From a Chemistry Transport Model. *J. Geophys. Res. Atmos.*, **126**, 2021.

43. James J. Krutak Robert D. Burpitt, William H. Moore, and John A. Hyatt, Chemistry of Ethenesulfonyl Fluoride. Fluorosulfonylethylation of Organic Compounds. *J. Org. Chem.*, **44**, 3847-3858, 1979.

44. Iimori H., Ando S., Shibasaki Y., Ueda M., Kishimura S., Endo M., Sasago M., A New Photoresist Materials for 157 nm Lithography-3: Poly[2-hydroxy-3-pinanyl vinyl sulfonate-*co*-4-(1, 1, 1, 3, 3, 3-hexafluoro-2-hydroxypropyl)styrene]. *J. Photopolym. Sci. Technol.*, **16**, 601-605, 2003.

45. Patrick J. F. A., Michel B., Jean-Charles B., Alain B., Yvette B., Marie-Annick C., Adam D., Gilles D., Claudine D. H., Marie-Hélène D., Jean M. D., Pierre G., Claude G., Eliane H., Bernard J,, Roselyne K., Sylvie G., Philippe H., Pierre M. L., Joseph L. B., Mireille M., Jean-Marie M., Conception N., Martine P., Odile P., Jeremy P., Jean R., Michel R., Jean-Marie S., Serge M., Riluzole Series. Synthesis and in Vivo "Antiglutamate" Activity of 6-Substituted-2-benzothiazolamines and 3-Substituted-2-imino-benzothiazolines. *J. Med. Chem.*, **42**, 2828-2843, 1999.

46. Aguilar B., Amissah F., Duverna R., Lamango N. S., Polyisoprenylation Potentiates the Inhibition of Polyisoprenylated Methylated Protein Methyl Esterase and the Cell Degenerative Effects of Sulfonyl Fluorides. *Curr. Cancer Drug Targets*, **11**, 752-762, 2011.

47. Kumara M. N., Nakahara T., Kobayashi S., Fujio M., Mishima M., Nucleophilicities of Alcohols and Water in Acetonitrile Based on Reactivities of Benzhydrylium Ions. *Bull. Chem. Soc. Jpn.*, **91**, 523-530, 2018.

48. Shinya M., Shinjiro K., Herbert M., Solvent Nucleophilicity. *J. Am. Chem. Soc.*, **126**, 5174-5181, 2004.

49. Schaller H. F., Tishkov A. A., Feng X., Mayr H., Direct Observation of the Ionization Step in Solvolysis Reactions: Electrophilicity versus Electrofugality of Carbocations. *J. Am. Chem. Soc.*, **130**, 3012-3022, 2008.

50. Mayer R. J., Breugst M., Hampel N., Ofial A. R., Mayr H., Ambident Reactivity of Phenolate Anions Revisited: A Quantitative Approach to Phenolate Reactivities. *J. Org. Chem*, **84**, 8837-8858, 2019.

CHAPTER 2

First GC/MS Identification of Aqueous Ammonia: Utilization of Ethenesulfonyl Fluoride as a Selective and Rapid Derivatization Reagent of Ammonia in Aqueous Media

2.1 Introduction

Ammonia is a colorless gas with a strong and distinctive pungent odor. It dissolves well in water, where it forms ammonium ions to make solutions alkaline.[1,2] Ammonia is used in a variety of applications, including as a refrigerant, a raw material for explosives, and an insect repellent.[3] In the field of forensic science, ammonia is an important analytical target because it is often misused for criminal purposes, such as contaminating food and drink, and as a corrosive substance in attacks against people and to damage cars.[4] Ammonia is toxic to humans. It induces respiratory arrest, causes corrosive inflammation in the gastrointestinal tract, and can cause blindness when it gets into the eyes.[5] In addition to criminal use, there is concern about accidents caused by refrigerant leaks. The identification of ammonia in forensic samples, including biological and environmental samples, is important for legal evidence. Most of the analytical targets are aqueous solutions containing ammonia, and there is a need for a rapid and simple method to identify ammonia in these solutions.

Various chromatographic techniques based on absorption have been used for quantitative analysis of ammonia in aqueous solutions, including IC,[6-9] GC,[10,11] and LC.[12-14] However, these methods cannot identify ammonia. Chromatographic techniques based on MS enable the identification of molecules using their unique masses.

Application of mass-based chromatography to ammonia requires derivatization of the ammonia because it is low molecular weight and high polarity are not suitable for GC/MS analysis.[15] To date, butyl chloroformate,[16,17] silylation reagents,[18,19] and acylation reagents[20,21] have been used for the derivatization of ammonia and amines in GC/MS analysis. However, these derivatization reagents cannot be used for aqueous ammonia because they are highly reactive with water. Thus, a cumbersome dehydration process is required before the derivatization of ammonia. A method using *ortho*-phthalaldehyde (OPA) to directly derivatize ammonia in aqueous solutions has been developed. However, it has been noted that the stability of ammonia-OPA derivatives is very poor.[13,14] Therefore, this method involves online derivatization of ammonia with OPA and detects ammonia-OPA derivatives.

Here, we investigated ESF as a derivatizing reagent for ammonia in aqueous media for GC/MS analysis. We found that ESF was a suitable derivatizing reagent for ammonia in aqueous media where there was a large excess of competitive water molecules. ESF rapidly produced tri-ESF derivatives (N-ESF_3) from ammonia that were suitable for separation by GC and mass detection (Fig. 2.1). We successfully applied this technique to the detection of ammonia in commercial products.

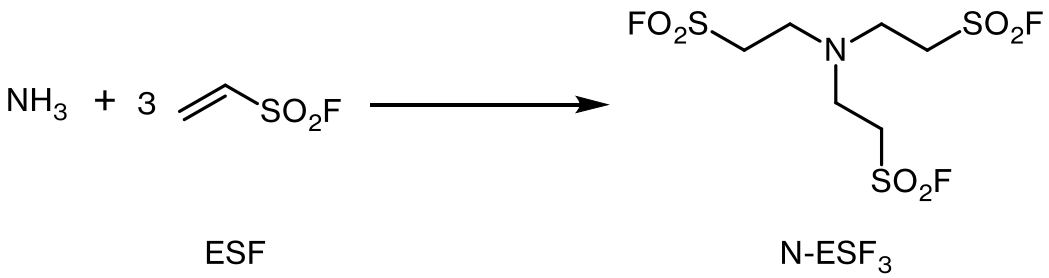


Fig. 2.1 The derivatization reaction of ammonia by ethenesulfonyl fluoride (ESF).

2.2 Experimental

2.2.1 Materials

A solution of 10% aqueous ammonia (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) was standardized by titration with sulfuric acid and diluted with pure water to prepare a 1000 μg/mL solution.[22] This stock solution was diluted with purified water to an arbitrary concentration. The derivatization reagent was prepared by dilution of ESF (Sigma-Aldrich, St. Louis, MO, USA) to a concentration of 5% by volume with methanol. Phenanthrene-d_{10} (FUJIFILM Wako Pure Chemical Corp.) was used as the internal standard (IS) and dissolved in ethyl acetate to a concentration of 1.0 μg/mL. Phenanthrene-*d*10 was selected as a substance with high stability and non-reactivity toward ESF in the IS selection process. The purified water used in this study was obtained from a Milli-Q Integral 5 system (Merck Millipore, Billerica, MA, USA). The stock solution was stored at 4°C. During the validation period, the same stock solution was used, and the obtained data showed little variation. The validation period lasted for 10 days.

2.2.2 Derivatization and Extraction Procedure

First, 100 μL of a sample containing ammonia was placed in a 1-mL test tube. Next, 100 μL of borate buffer (pH 9.0) and 100 μL of 600 mM methanol solution of ESF were added, and the resulting solution was vortexed for 30 sec at room temperature before heating at 80°C for 10 min. Then, 100 μL of ethyl acetate containing IS was added to the solution, vortexed for 30 sec at room temperature, and centrifuged at 2180 ×*g* for 3 min. The organic layer was placed in a separate glass tube, and 1 μL of this solution was injected into the GC/MS.

2.2.3 GC/MS Analysis

GC/MS determination was performed on a GCMS-QP2020 NX (Shimadzu, Kyoto, Japan) equipped with a DB-17 MS capillary column (30 m × 0.25 mm *i.d.*, 0.25 μm film thickness, Agilent Technologies, Santa Clara, CA). The GC oven temperature was held at 140°C for 1 min and increased to 320°C at 20°C/min (held for 1 min). The injection mode was splitless and the carrier gas was helium at a flow rate of 11.9 mL/min and an injection volume of 1.0 μL. The MS interface temperature was 250°C and the ion source temperature was 250°C. The system was operated with a filament current of 150 μA, an electron energy of 70 eV, and in electron ionization mode. The derivative was identified in full scan mode, and data were acquired from *m/z* 45 to 400. Quantitation was performed in selective ion monitoring mode with monitoring of ions at *m/z* <u>250</u> and 111 for N-ESF$_3$ and *m/z* <u>188</u> and 160 for the IS (the quantitation ions are underlined).

2.2.4 IC Analysis

IC determination was performed on an ICS-2100 (DIONEX, Sunnyvale, CA, USA) equipped with an electric conductivity detector, an IonPac CS12A column (250 mm × 4 mm *i.d.*, Thermo Fisher Scientific, Waltham, MA, USA), IonPac CG12A guard column (50 mm × 4 mm *i.d.*, Thermo Fisher Scientific), and Dionex CERS 500 (4 mm, Thermo Fisher Scientific) as the suppressor. The mobile phase consisted of 20 mM methane sulfonic acid (Thermo Fisher Scientific). The IC column oven temperature was 35°C, with a flow rate of 1 mL/min and an injection volume of 25 μL.

2.3 Results and Discussion

2.3.1 Optimization of Ammonia Derivatization by ESF

First, we examined the applicability of ESF to the derivatization of ammonia in water. The derivatization reaction was performed by mixing 100 μL of aqueous ammonia (10.0 μg/mL, 0.6 mM), 100 μL of borate buffer (pH 9.0), and 100 μL of a methanol solution of ESF (600 mM) for 10 min at 80°C. ESF was found to be handled as a methanol solution because of the negligible reactivity of ESF in methanol at least for 6 months. The amount of ESF was 1000 times that of ammonia. The reactants were extracted with ethyl acetate and analyzed on a mid-polarity GC column using phenanthrene-d_{10} as an internal standard. We successfully detected the di-ESF derivative (NH-ESF_2) and N-ESF_3 in the extracted ion chromatograms (Fig. 2.2 A). In addition to the molecular ion peak at *m/z* 237, the mass spectrum of NH-ESF_2 showed a base peak at *m/z* 140 which corresponds to the loss of -CH_2SO_2F (Fig. 2.2 B). The mass spectrum of N-ESF_3 showed a molecular ion peak at *m/z* 347 and a base peak at *m/z* 250 corresponding to the loss of -CH_2SO_2F (Fig. 2.2 C). The molecular ion peak at *m/z* 188 was observed for phenanthrene-*d*10 (Fig. 2.2 D). The mono-ESF derivative was not detected, even with a very short reaction time, which showed that the mono-ESF derivative rapidly reacted with ESF to produce multi-ESF derivatives. Because ammonia has much higher nucleophilicity than water,[23,24] the large excess of water molecules in aqueous ammonia does not affect the derivatization reaction of ammonia by ESF.

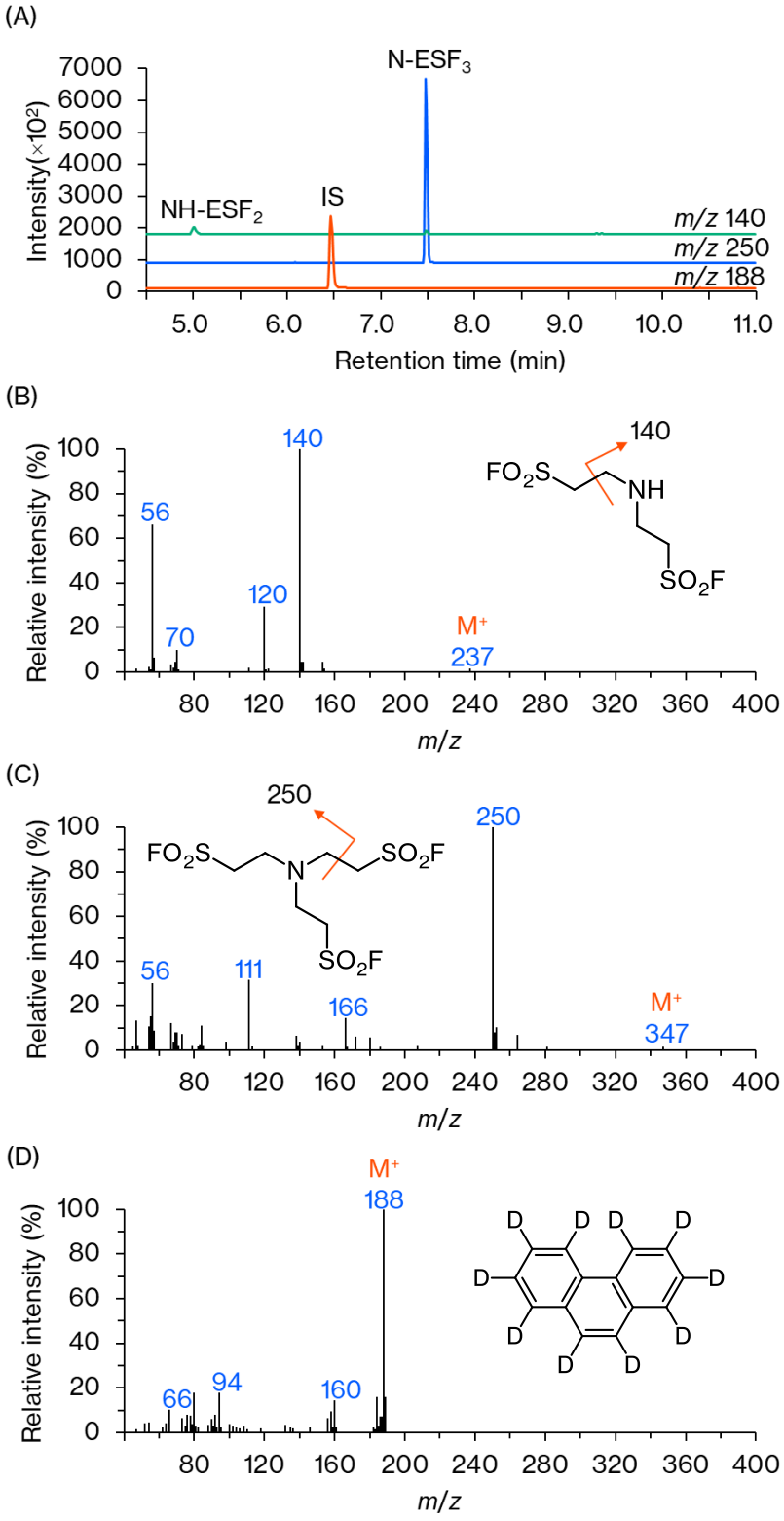


Fig. 2.2 Extracted ion chromatograms of the ESF-derivatized ammonia (A). Mass spectra of NH-ESF_2 (B), N-ESF_3 (C), and the IS (D).

2.3.2 Stability

To evaluate the stability of N-ESF$_3$ after extraction into ethyl acetate, samples were analyzed during storage at room temperature (Fig. 2.3). No loss of ammonia derivatives was detected until 24 h of storage, which showed that N-ESF$_3$ had good stability in ethyl acetate.

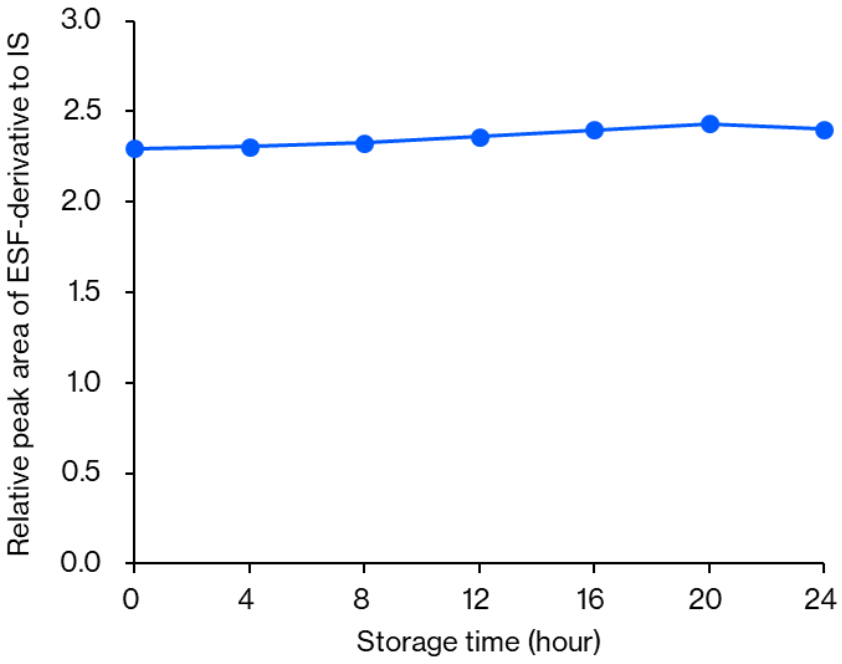


Fig. 2.3 Effect of storage time of the ethyl acetate extract of the derivatized reaction solution on the GC/MS analysis. The extract was stored at room temperature before the analysis.

2.3.3 Derivatization Temperatures & Times

We examined effect of reaction temperature for the ammonia derivatization. Fig. 2.4 shows the time-dependent change of the amount of NH-ESF_2 and N-ESF_3 derivatives at different reaction temperatures. The amount of N-ESF_3 increased at higher reaction temperatures. The time-dependent increase of N-ESF_3 signal was observed at room temperature and 50°C while it was saturated from the beginning (10 min) at 80°C. Hereafter, 80°C for 10 min was used as the derivatization reaction condition.

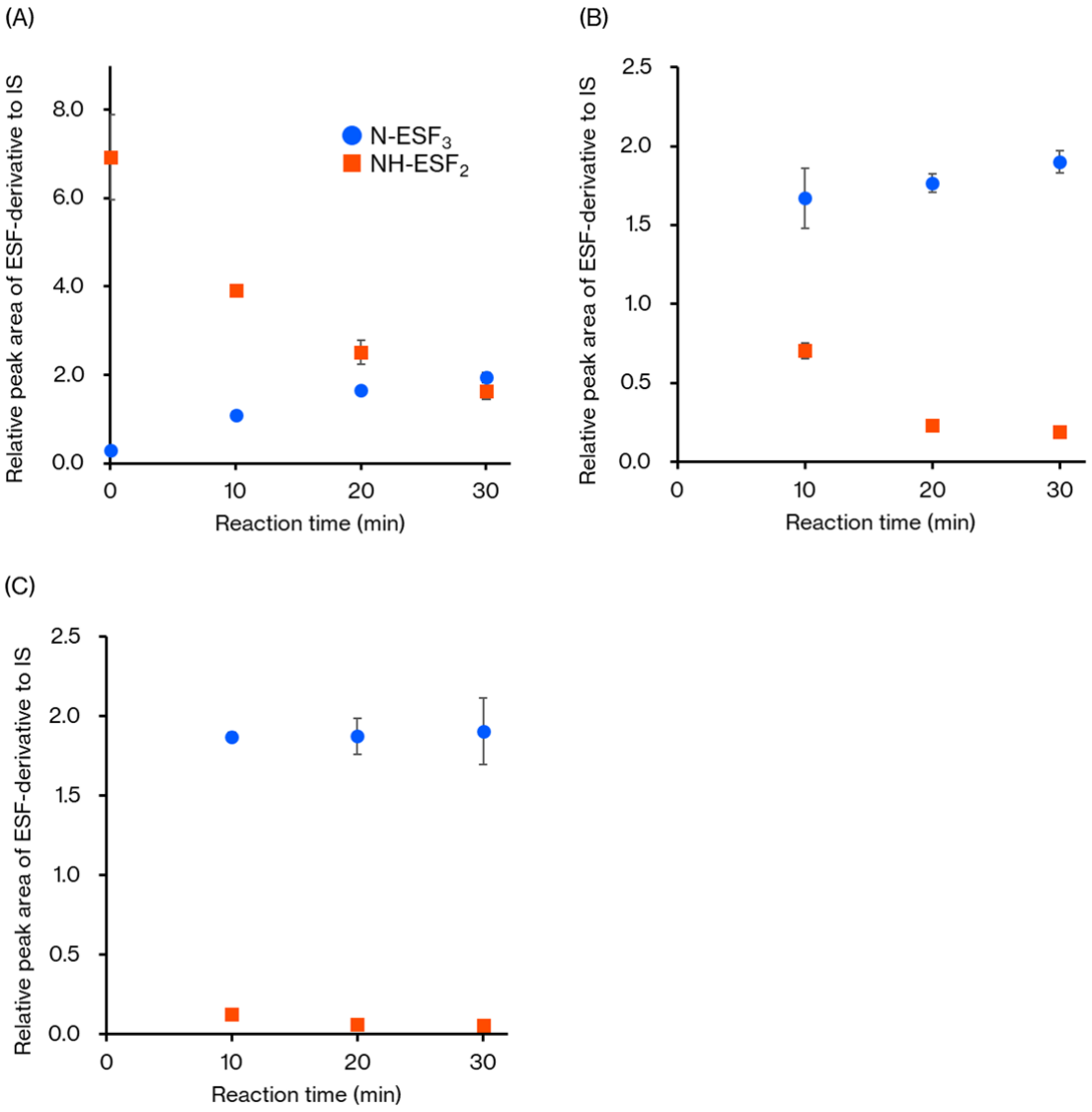


Fig. 2.4 Effect of the reaction temperature on ammonia derivatization. Each experiment was performed three times, and the mean value was presented with the SD. The results for ammonia-ESF derivatives are represented in the following panels: room temperature (A), 50°C (B), and 80°C (C).

2.3.4 Effect of pHs

The effect of the buffer pH on the derivatization of ammonia was studied using the following five buffers that do not react with ESF: phthalic acid ($C_8H_5KO_4$), potassium dihydrogen phosphate (KH_2PO_4), boric acid (H_3BO_3), disodium hydrogen phosphate (Na_2HPO_4), and potassium chloride (KCl) (Table 2.1). The derivatization reaction will proceed more rapidly with neutral ammonia than ammonium and ammonia will be readily evaporated, leading to loss of ammonia before derivatization. Thus, we expected that the efficacy of the derivatization would be changed at the solution pH around the p*K*a of the ammonium ion (9.25); however, the results were not as simple as we expected (Fig. 2.5).[25] The amount of N-ESF_3 was high at low and high pH ranges ($pH \leq 7.0$ and ≥ 11.0); however, the precision was also low, which showed that there was high variability in these pH ranges (Table 2.1). The obtained intensity has increased significantly, but along with it, the uncertainty has also increased. By contrast, at moderate pH ($8.0 \leq pH \leq 10.0$), the amount of N-ESF_3 was low and the coefficient of variation was very low, especially at pH 8.0 and 9.0. Thus, pH 9.0 was selected as the optimum condition for derivatization because it provided sufficient sensitivity with low variability.

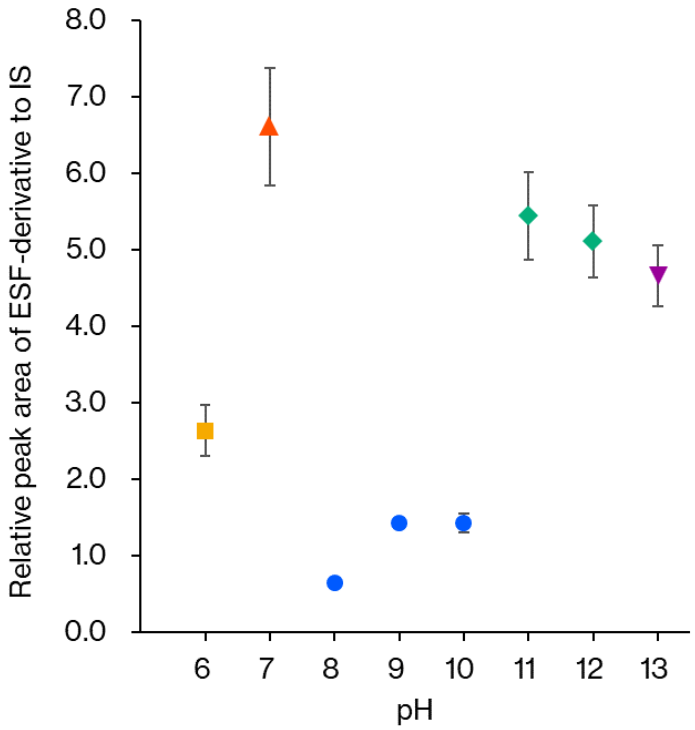


Fig. 2.5 Effect of the buffer solution pH on derivatization. Each operation was performed three times and the mean value is presented with SD. The results for different buffers are shown using the following colors: $C_8H_5KO_4$/NaOH (■, yellow), KH_2PO_4/NaOH (▲, red), H_3BO_3/KCl/NaOH (●, blue), Na_2HPO_4/NaOH (◆, green), and KCl/NaOH (▼, purple).

Table 2.1 Effects of pH on ammonia derivatization.

pH	Buffer solutions	Mean ± SD	Precision (% RSD) [a]
6.0	0.1 mol/L $C_8H_5KO_4$/NaOH	2.64 ± 0.33	12.5
7.0	0.1 mol/L KH_2PO_4/NaOH	6.62 ± 0.77	11.6
8.0	0.1 mol/L H_3BO_3/KCl/NaOH	0.65 ± 0.01	0.4
9.0	0.1 mol/L H_3BO_3/KCl/NaOH	1.43 ± 0.03	2.4
10.0	0.1 mol/L H_3BO_3/KCl/NaOH	1.43 ± 0.12	8.7
11.0	0.05 mol/L Na_2HPO_4/NaOH	5.45 ± 0.57	10.5
12.0	0.05 mol/L Na_2HPO_4/NaOH	5.12 ± 0.47	9.2
13.0	0.2 mol/L KCl/NaOH	4.66 ± 0.39	8.5

[a] Precision (% relative standard deviation) was defined as (standard deviation/mean peak area ratio to the IS) × 100.

2.3.5 Effect of Extraction Solvents

The effects of extraction solvents on the amount of N-ESF$_3$ were examined using three solvents (*n*-hexane, toluene, and ethyl acetate). *n*-Hexane could not extract N-ESF$_3$ at all, whereas toluene and ethyl acetate efficiently extracted N-ESF$_3$ (Fig. 2.6). These results showed that the higher polarity solvents were more suitable for the extraction of N-ESF$_3$, which will have polar nature due to sulfonyl fluoride groups. We selected ethyl acetate as the optimum extraction solvent.

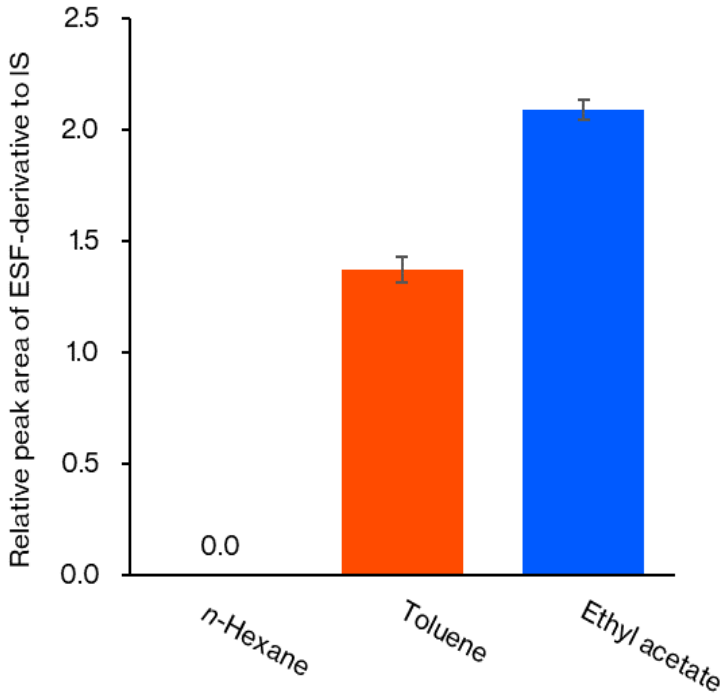


Fig. 2.6 Effect of different extraction solvents for the derivatized sample. Each operation was performed three times and the mean value is presented with SD.

2.4 Analytical Ability

A calibration curve was prepared using solutions with ammonia concentrations ranging from 0.10 to 100.0 μg/mL (Fig. 2.7). The linearity of the calibration curve was evaluated using the relationship between the signal intensity of N-ESF_3 and the concentration of ammonia. The area ratio of the base peak at *m/z* 250 for N-ESF_3 to the base peak at *m/z* 188 for the IS was plotted against the concentration of ammonia. Linear regression analysis with a weighting factor of 1/*x* showed good linearity (R^2 = 0.9998).

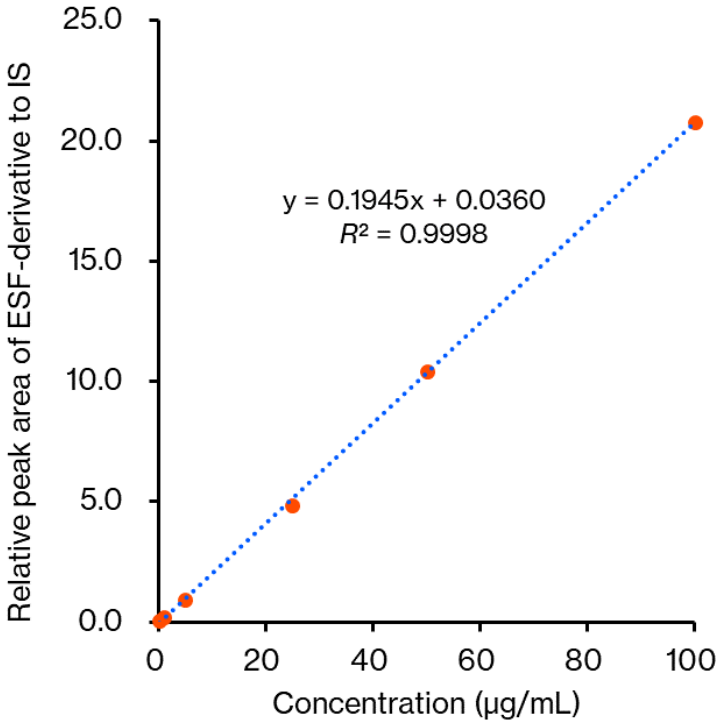


Fig. 2.7 Calibration curve showing the peak areas for N-ESF_3 generated from ammonia concentrations ranging from 0.10 to 100.0 μg/mL.

Water samples adjusted to four concentrations (0.10, 0.25, 10.0, and 75.0 μg/mL) were analyzed five times each and the analyses were repeated for 3 days to evaluate intra- and inter-day precision and accuracy (Table 2.2). % RSD and % bias for intra- and inter-day met the criteria of the pharmaceutical analysis method.[26,27] The limit of detection based on a signal-to-noise (S/N) ratio of 3 was 0.05 μg/mL, and the limit of quantification based on a S/N ratio of 10 was 0.10 μg/mL. The results showed that the method was very stable and highly reproducible.

Table 2.2 Intra- and inter-day precision and accuracy.

Nominal concentration (μg/mL)	Intra-day (n = 5)			Inter-day (n = 3)		
	Mean ± SD	Precision (% RSD) [a]	Accuracy (% bias) [b]	Mean ± SD	Precision (% RSD) [a]	Accuracy (% bias) [b]
0.1	0.10 ± 0.01	7.9	0.9	0.11 ± 0.01	4.2	5.3
0.25	0.25 ± 0.02	7.2	0.5	0.26 ± 0.01	2.3	3.7
10	10.2 ± 0.5	5.0	1.6	10.6 ± 0.3	3.1	6.0
75	80.3 ± 6.1	7.6	7.1	77.8 ± 1.8	2.4	3.7

[a] Precision (% relative standard deviation) was defined as (standard deviation/mean peak area ratio to the IS) × 100.

[b] Accuracy (% bias) was defined as [(measured concentration − nominal concentration)/nominal concentration] × 100.

2.5 Interference

Potential interference from seven substances was investigated (Table 2.3). The amino acids and glucose were selected as potential contents in beverages in case of the criminal usage of ammonia to add in beverages. Sodium hypochlorite which is a readily available toxic compound was chosen in case of mixed usage with ammonia. Dimethylamine was selected as a representative alkylamine. Mixed solutions containing these substances (0.1, 1.0, or 10.0 mol equivalents relative to ammonia) were prepared with a constant ammonia concentration of 10.0 μg/mL. The recovery was determined by comparing the signal intensity obtained in the mixed solution with that obtained in the additive-free solution. The results showed that hypochlorous acid greatly reduced the signal intensity of N-ESF$_3$. This was likely caused by consumption of ammonia by its reaction with hypochlorite to produce chloramines.[28] The signal intensity of N-ESF$_3$ decreased with dimethylamine when a 10-fold excess was added. This may have been caused by the much higher nucleophilicity of dimethylamine (approximately 10^7 times) compared with that of ammonia,[23] leading to the consumption of ESF by dimethylamine. By contrast, amino acids (glycine, valine, aspartic acid, and ornithine) and glucose had no effect on ammonia derivatization. Notably, the α-amine group of these amino acids and the δ-amine group of ornithine did not disturb the ammonia detection even when a 10-fold excess was added.

Table 2.3 Effects of substances that could interfere with ammonia determination.

	Recovery [a] Equivalent relative to ammonia [b]		
Additives	0.1	1.0	10.0
hypochlorous acid	8.5 ± 0.05	8.0 ± 0.02	1.4 ± 0.02
dimethylamine	10.2 ± 0.16	9.9 ± 0.04	7.8 ± 0.21
L-valine	9.3 ± 0.22	10.2 ± 0.24	9.5 ± 0.13
DL-ornithine	9.4 ± 0.30	10.1 ± 0.11	9.6 ± 0.11
glucose	9.6 ± 0.10	10.1 ± 0.07	9.8 ± 0.16
DL-aspartic acid	10.0 ± 0.20	10.0 ± 0.14	9.8 ± 0.06
glycine	9.2 ± 0.08	10.8 ± 0.51	10.7 ± 0.41

[a] Mean ± standard deviation (n = 3).

[b] Each additive was added at 0.1, 1.0, or 10.0 mol equivalents relative to ammonia.

2.6 Practical Application

To verify the reliability of this method, four commercially available samples containing high concentrations of ammonia were analyzed. Samples A and B were respiratory stimulants, and samples C and D were antipruritic agents (Table 2.4). Samples C and D contained additives such as menthol and salicylic acid. Samples A, B, C, and D were diluted with distilled water. Direct detection of ammonia by IC, which is approved by Japanese Industrial Standards, was used to validate our method.[22] Both methods showed similar concentrations for each sample, and the correlation coefficient of the two methods was 0.997, which indicated that our method was highly reliable and not affected by the high concentrations of additives in commercial samples. The conventional IC method can only quantify ammonia, but our method can both quantify and identify ammonia, which meets the requirements for using forensic samples as legal evidence.

Table 2.4 Results for ammonia quantification in commercial samples.

Commercial samples	IC [a,b] $\times 10^3$ μg/mL	Proposed method [b] $\times 10^3$ μg/mL
A	97.3 ± 0.01	99.8 ± 0.5
B	103.8 ± 0.01	101.6 ± 0.1
C	21.5 ± 0.01	20.5 ± 0.1
D	24.1 ± 0.01	21.1 ± 0.2

[a] Values measured by the established IC method.[22]

[b] Mean ± standard deviation (n = 3).

2.7 Summary

We developed a GC/MS method for rapid and selective analysis of ammonia in aqueous media. We found that ESF was a suitable derivatizing reagent for ammonia in aqueous media. ESF could selectively react with ammonia even in the presence of the excess amount of amine- and hydroxyl-containing interfering substances. The method was successfully applied to ammonia quantification in commercial samples. With its high specificity and good reliability, this method will be useful in many analytical fields, including forensic science.

2.8 References

1. P. Warneck, *Chemistry of the natural atmosphere*, Academic Press, 2nd ed., 2000.
2. Liu L., Yang Y., Xi R., Zhang X., Xu W., Liu X., Li Y., Liu P., Wang Z., Global Wet-Reduced Nitrogen Deposition Derived From Combining Satellite Measurements With Output From a Chemistry Transport Model, *JGR: Atmospheres*, **126**, 2021.
3. Behera S. N., Sharma M., Aneja V. P., Balasubramanian R., Ammonia in the atmosphere: a review on emission sources, atmospheric chemistry and deposition on terrestrial bodies. *Environ Sci Pollut Res Int*, 2013, **20**, 8092-8131.
4. Thierry D., Bozec N. L., Persson D., Corrosion of hot-dip-galvanised steel and zinc alloy-coated steel in ammonia and ammonium chloride. *Mater. Corros.*, **71**, 1118-1124, 2020
5. Organization World Health, Environmental Health Criteria 54. https://www.inchem.org/documents/ehc/ehc/ehc54.htm, accessed Jul 3, 2022.
6. Bouyoucos. S. A., Determination of Ammonia and Methylamines in Aqueous Solutions by Ion Chromatography. *Anal. Chem.*, **49**, 401-403, 1977.
7. Pohl. C, Rey M., Jensen D., Kerth J., Determination of sodium and ammonium ions in disproportionate concentration ratios by ion chromatography. *J. Chromatogr. A*, **850**, 239-245, 1999.
8. Rey M. A., Pohl C. A., Jagodzinski J. J., Kaiser E. Q. Riviello J. M., A new approach to dealing with high-to-low concentration ratios of sodium and ammonium ions in ion chromatography. *J. Chromatogr. A*, **804**, 201-209, 1998.

9. Steinkamp F. L., Giordano B., Collins G., Rose-Pehrsson S., Volatile Emissions of Ammonium Nitrate under Flowing Conditions. *Propellants, Explosives, Pyrotechnics*, **40**, 682-687, 2015.

10. Jenkins R. W.,Cheek C. H., Linnenbom V. J., Determination of Dissolved Ammonia by Gas Chromatography. *Anal. Chem.*, **38**, 1257-1258, 1966.

11. Zaffaroni R., Ripepi D., Middelkoop J., Mulder F. M, Gas Chromatographic Method for In Situ Ammonia Quantification at Parts per Billion Levels. *ACS Energy Letters*, **5**, 3773-3777, 2020.

12. Sahasrabuddhey B., Jain A., Verma K. K., Determination of ammonia and aliphatic amines in environmental aqueous samples utilizing pre-column derivatization to their phenylthioureas and high performance liquid chromatography. *Analyst*, **124**, 1017-1021, 1999.

13. Need A., Karmen C., Sivakoff S., Karmen A., Specific detection of nitrogen in gas—liquid chromatographic effluents by fluorescent detection of ammonia. *J. Chromatogr. A*, **158**, 153-160, 1978.

14. Huang G., Hou J., Zhou X., A Measurement Method for Atmospheric Ammonia and Primary Amines Based on Aqueous Sampling, OPA Derivatization and HPLC Analysis. *Environ. Sci. Technol.*, **43**, 5851-5856, 2009.

15. Kranenburg R. F., Verduin J., Stuyver L. I., Ridder R., Beek A., Colmsee E., Asten A. C., Benefits of derivatization in GC–MS-based identification of new psychoactive substances. *Forensic Chem.*, **20**, 100273, 2020.

16. Lubrano A. L., Andrews B., Hammond M., Collins G. E., Rose-Pehrsson S., Analysis of ammonium nitrate headspace by on-fiber solid phase

microextraction derivatization with gas chromatography mass spectrometry. *J. Chromatogr. A*, **1429**, 8-12, 2016.

17. Katilie C. J., Simon A. G., DeGreeff L. E., Quantitative analysis of vaporous ammonia by online derivatization with gas chromatography - mass spectrometry with applications to ammonium nitrate-based explosives. *Talanta*, **193**, 87-92, 2019.

18. Sobolevsky T. G., Revelsky A. I., Miller B., Oriedo V., Chernetsova E. S., Revelsky I. A., Comparison of silylation and esterification/acylation procedures in GC-MS analysis of amino acids. *J. Sep. Sci.*, **26**, 1474-1478, 2003.

19. Nishio T., Toukairin Y., Hoshi T., Arai T., Nogami M., Determination of 3-chloro-l-tyrosine as a novel indicator of chlorine poisoning utilizing gas chromatography-mass spectrometric analysis. *Leg Med (Tokyo)*, **47**, 101782, 2020.

20. Zampolli M., Meunier D., Sternberg R., Raulin F., Szopa C., Pietrogrande M. C., Dondi F., GC-MS analysis of amino acid enantiomers as their N(O, S)-perfluoroacyl perfluoroalkyl esters: application to space analysis. *Chirality*, **18**, 279-295, 2006.

21. Kusch P., Knupp G., Hergarten M., Kozupa M., Majchrzak M., Identification of corrosion inhibiting long-chain primary alkyl amines by gas chromatography and gas chromatography–mass spectrometry. *Int. J. Mass Spectrom.*, **263**, 45-53, 2007.

22. Japanese Standards Association, Testing Methods for Industrial Wastewater, JIS K0102, https://www.jisc.go.jp/index.html, 2019, 1, pp. 150-159, accessed May 5, 2022.

23. Brotzel F., Cheung Y. Mayr H., Nucleophilicities of Primary and Secondary Amines in Water. *J. Org. Chem.*, **72**, 3679–3688, 2006.

24. Minegishi S., Kobayashi S., Mayr H., Solvent Nucleophilicity. *J. Am. Chem. Soc.*, **126**, 5174-5181, 2004.

25. Zhang M., Dong X., Li X., Jiang Y., Li Y., Liang Y., Review of separation methods for the determination of ammonium/ammonia in natural water. *Trends in Environmental Anal. Chem.*, **27**, 2020.

26. European Medicines Agency (EMA), ICH M10 on bioanalytical method validation - Scientific guideline, https://www.ema.europa.eu/en/documents/scientific-guideline/ich-guideline-m10-bioanalytical-method-validation-step-5_en.pdf., accessed Jul 15, 2022.

27. Food and Drug Administration (FDA), M10 Bioanalytical Method Validation and Study Sample Analysis, https://www.fda.gov/media/162903/download., accessed Jul 15, 2022.

28. Pressley T. A., Ammonia-Nitrogen Removal by Breakpoint Chlorination. *Environ. Sci. Technol.*, **6**, 622-628, 1972.

CHAPTER 3

GC/MS-Based Quantitative Analysis of Sulfide Ion in Whole Blood Using Ethenesulfonyl Fluoride as a Derivatization Reagent

3.1 Introduction

Hydrogen sulfide (H_2S) is a gas with a rotten egg smell and is naturally emitted from volcanic regions, hot springs, decomposition of rotting organic matter, and paper mills, among other sources.[1-5] It is known to have a dual nature in biological systems. Beneficial effects include neural and cellular protection in the body, anti-epileptic effects, and potential in diabetes treatment.[6-8] However, inhalation at high concentrations is toxic, leading to frequent incidents of poisoning in areas where H_2S is generated.[4,9] In the poisoning cases, there are instances where the poisoning occurs through the ingestion of pesticides containing sulfides, or by inhaling H_2S gas produced by mixing such pesticides with acidic cleaning agents.[10,11] Therefore, H_2S is an important analyte in forensic chemistry for investigating the causes of incidents and accidents. It is also known that H_2S decomposes in the presence of oxygen in the blood,[12,13] rapid methods are required for the detection of H_2S in biological samples.

As such rapid methods, colorimetric methods,[14-16] GC,[17-21] and LC [22,23] have been developed so far. However, information obtained by these methods, such as absorbance or retention time, lack specificity to identify sulfide ion. Mass detection of sulfide ion by GC/MS is more specific than these methods.[4,24] To improve the specificity of mass identification of sulfide ion, its derivatization is useful because of the enlargement

of molecular weight and specific pattern of the mass of fragmented compounds. PFBBr has been used for the derivatization of sulfide ion. However, the hydrophobic nature of this reagent increases the deviation of the result and the tear-gas-like irritating nature requires careful handling.[24,25]

Here we proposed ESF as a derivatizing reagent for the analysis of sulfide ion present in whole blood by GC/MS. We found here that ESF rapidly reacted with sulfide ion in whole blood and formed a suitable di-ESF derivative (S-ESF_2) that can be separated and detected by GC/MS (Fig. 3.1).

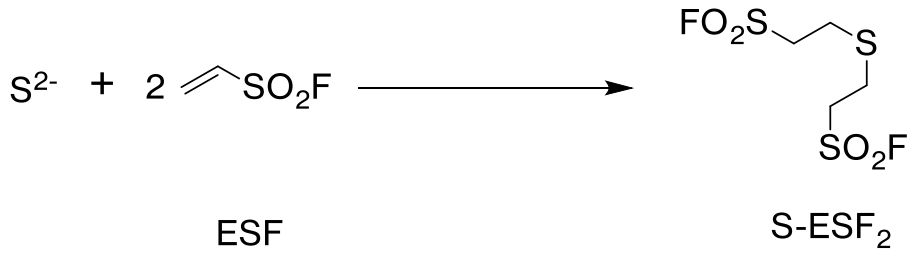


Fig. 3.1 The derivatization reaction of sulfide ion by ethenesulfonyl fluoride (ESF).

3.2 Experimental

3.2.1 Materials

Sodium sulfide (FUJIFILM Wako Pure Chemical Corp.) was dissolved in water and titrated.[26] The titrated solution was prepared at a concentration of 1000 μg/mL to create a standard sulfide ion solution. This standard sulfide ion solution was diluted with water to the desired concentrations and added to the blood as samples. The derivatization reagent was prepared by diluting ESF (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) to a concentration of 5% by volume with acetone. Phenanthrene-*d*10 (FUJIFILM Wako Pure Chemical Corp.) was used as the IS and dissolved in acetone to a concentration of 1.0 μg/mL. To prevent the decomposition of H_2S due to dissolved oxygen,[12,13] the water and organic solvents used in the experiment were bubbled with nitrogen to remove oxygen.[24] The purified water used in this study was obtained from a Milli-Q Integral 5 system (Merck Millipore). The commercially available human whole blood was purchased (Cosmo Bio Corp., Tokyo, Japan).

3.2.2 ESF Derivatization and Extraction Procedure

First, 100 μL of a whole blood sample containing sulfide ion was added to a 1.5-mL plastic tube. Next, 100 μL of borate buffer solution (pH 8.0), 100 μL of acetone solution containing IS, and 100 μL of acetone solution containing ESF at a concentration of 600 mM, and 100 μL of *n*-hexane were added to the tube. The mixture was vortexed at room temperature for 1 min. The tube was then centrifuged at 2000 × *g* for 1 min. The organic layer was placed in a separate glass tube, and 1.0 μL of this solution was injected into the GC/MS.

3.2.3 HS-GC/MS Analysis Procedure

The analysis using HS-GC/MS was conducted with reference to prior reports.[25] In a glass vial with a volume of 20-mL, 500 μL of whole blood sample containing sulfide ion and 500 μL of an aqueous solution containing 0.01 vol% of methanol as IS were added and mixed. To this solution, 1000 μL of acetone was added and vortexed for 5 sec. Subsequently, 500 μL of phosphoric acid (FUJIFILM Wako Pure Chemical Corp.) was added, the vial was quickly sealed, and it was vortexed for 5 sec. Afterward, the analysis was performed using HS-GC/MS.

3.2.4 PFB Derivatization and Extraction Procedure

The method for derivatization GC/MS using PFBBr was adapted from previously reported techniques.[4] In a 10-mL test tube, 0.8 mL of 5 mmol/L tetradecyldimethylbenzylammonium (TDMBA) chloride (Tokyo Chemical Industry Co., Ltd.) aqueous solution, 0.5 mL of 20 mmol/L PFBBr (Sigma-Aldrich) in ethyl acetate, and 2.0 mL of 1.0 μmol/L 1,3,5-tribromobenzene (TBB) (FUJIFILM Wako Pure Chemical Corp.) in ethyl acetate as an IS were added. To this solution, 200 μL of blood sample containing sulfide ion was added and then vortexed for 1 min. Then, 0.10 g of potassium dihydrogen phosphate (FUJIFILM Wako Pure Chemical Corp.) was added, and the solution was vortexed for another 10 sec. The test tube was then centrifuged at 2000 × *g* for 1 min. The organic layer was transferred to a separate glass container, and 1.0 μL of it was analyzed using GC/MS.

3.2.5 GC/MS Analysis (ESF Derivatization)

GC/MS determination was performed on a GCMS-QP2020 NX (Shimadzu) equipped with a DB-5 MS capillary column (30 m × 0.25 mm *i.d.*, 0.25 μm film thickness, Agilent Technologies). The GC oven temperature was held at 80°C for 1 min and increased to 320°C at 20°C/min (held for 2 min). The injection mode was splitless and the carrier gas was helium at a flow rate of 20.2 mL/min and an injection volume of 1.0 μL. The MS interface temperature was 250°C and the ion source temperature was 250°C. The system was operated with a filament current of 150 μA, an electron energy of 70 eV, and in EI mode. The derivative was identified in full scan mode, and data were acquired from *m/z* 30 to 300. Quantitation was performed in SIM mode, monitoring ions at *m/z* 86, 170, and 254 for S-ESF_2 and *m/z* 160, and 188 for the IS (the quantitation ions are underlined).

3.2.6 HS-GC/MS Analysis

HS-GC/MS determination was performed on a GCMS-QP2020 NX instrument (Shimadzu) equipped with a Pora PLOT Q-HT capillary column (25 m × 0.32 mm *i.d.*, 10 μm film thickness, Agilent Technologies). An HS-20 headspace autosampler (Shimadzu) was used. The oven temperature for the headspace autosampler was set at 60°C, the sample line temperature at 100°C, and the transfer line temperature at 150°C. The vial equilibration time was 10 min, and helium was used as the vial pressurization gas with a vial pressurization time of 0.5 min, and a pressurization equilibration time of 0.1 min. The loading time was 0.5 min with no loading equilibration time, injection time was 0.5 min, and the needle flush time was 5 min. The GC oven temperature was initially held at 40°C for 1.5 min, then increased to 250°C at a rate of 25°C/min (held for 1 min).

The injection mode was set to split (split ratio 1:5), and helium was used as the carrier gas with a flow rate of 11.6 mL/minute. The MS interface temperature was 230°C, and the ion source temperature was 200°C. The system was operated with a filament current of 150 μA, an electron energy of 70 eV, and in EI mode. Hydrogen sulfide was quantified in SIM mode, monitoring ions at *m/z* 34 and 33 for H_2S, and *m/z* 31 and 29 for the IS (quantification ions are underlined).

3.2.7 GC/MS Analysis (PFB Derivatization)

GC/MS determination was performed on a GCMS-QP2020 NX (Shimadzu) equipped with a DB-5 MS capillary column (30 m × 0.25 mm *i.d.*, 0.25 μm film thickness, Agilent Technologies). The GC oven temperature was held at 100°C for 2 min and increased to 290°C at 10°C/min (held for 5 min). The injection mode was splitless and the carrier gas was helium at a flow rate of 10.7 mL/min and an injection volume of 1.0 μL. The MS interface temperature was 250°C and the ion source temperature was 230°C. The system was operated with a filament current of 150 μA, an electron energy of 70 eV, and in EI mode. The derivative was identified in full scan mode, and data were acquired from *m/z* 30 to 500. Quantitation was performed in SIM mode, monitoring ions at *m/z* 181, and 394 for S-PFB_2 and *m/z* 235, and 314 for the IS (the quantitation ions are underlined).

3.3 Results and Discussion

3.3.1 Optimization of Sulfide Ion Derivatization by ESF

We examined derivatization of sulfide ion spiked in whole blood by ESF. The blood concentration of sulfide ion is reported to be below 0.05 μg/mL, while in cases of fatal poisoning due to H_2S exposure, it has been reported to range from 0.32 to 2.56 μg/mL.[4,27,28] 100 μL of whole blood sample spiked with sulfide ion (1.0 μg/mL, 0.03 mM) was mixed with 100 μL of borate buffer solution (pH 8.0), 100 μL of acetone solution of phenanthrene *d*-10 (1.0 μg/mL) as an internal standard, 100 μL of acetone solution of ESF (600 mM) and 100 μL of *n*-hexane which are poor solvent for blood proteins were added. Then the resulting dispersion was vortexed at room temperature for 1 min. ESF was present at approximately 20,000 times the concentration of sulfide ion in the mixed solution. After the reaction, the dispersion was centrifuged for precipitation of protein fractions and separation of organic and aqueous phases, then the organic *n*-hexane layer was analyzed using a low-polarity column. As shown in Fig. 3.2, the extracted ion chromatogram revealed the presence of S-ESF_2 (peaks at *m/z* 254 and 86 are assignable to a molecular ion and a decomposed product). It is notable that mono-ESF derivative was not detected in the mass chromatogram even with very short reaction time, 1 min. This suggests that mono-ESF derivative rapidly react with ESF to form S-ESF_2.

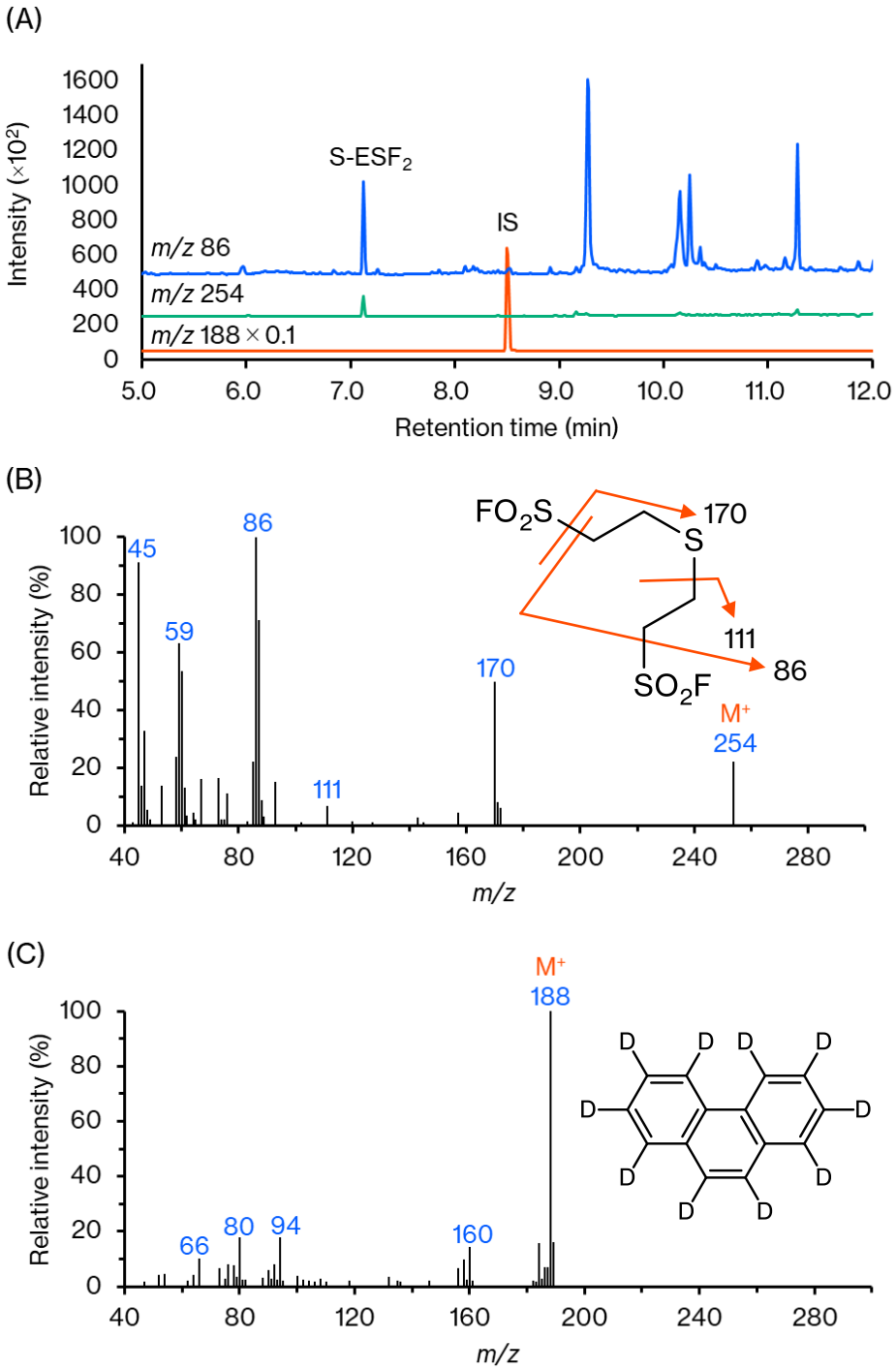


Fig. 3.2 Extracted ion chromatograms of the ESF-derivatized sulfide ion (A). Mass spectra of S-ESF_2 (B), and the IS, phenanthrene *d*-10 (C). Components with *m/z* 86, 254, and 188 detected in panel A were assignable to fragment ion of S-ESF_2, molecular ion of S-ESF_2, and molecular ion of IS.

3.3.2 Stability

We evaluated the stability of S-ESF$_2$ extracted into the *n*-hexane phase during the storage at room temperature (Fig. 3.3). No loss of S-ESF$_2$ was observed for at least 36 hours. Considering that easily oxidized property and high volatility of sulfide ion, the high stability of S-ESF$_2$ is advantageous for practical analysis.

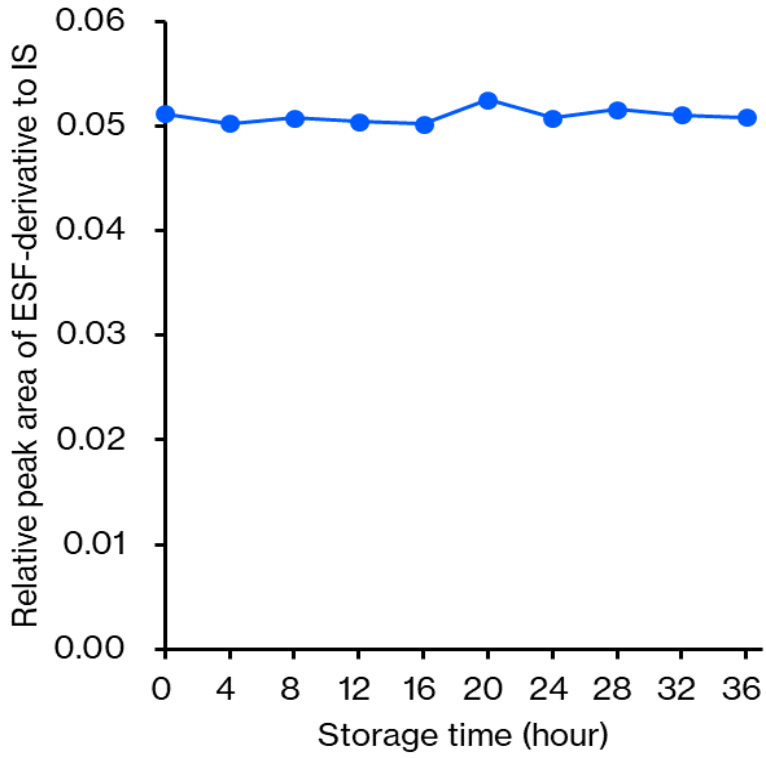


Fig. 3.3 Effect of storage time of the *n*-hexane extract of the derivatized reaction solution on the GC/MS analysis. The extract was stored at room temperature before the analysis.

3.3.3 Derivatization Timings

In method 1, derivatization of sulfide ion and protein precipitation occurred simultaneously. We compared efficacy of derivatization protocol of sulfide ion with method 2 in which the derivatization was performed to the supernatant after precipitation of proteins in the blood (Fig. 3.4).

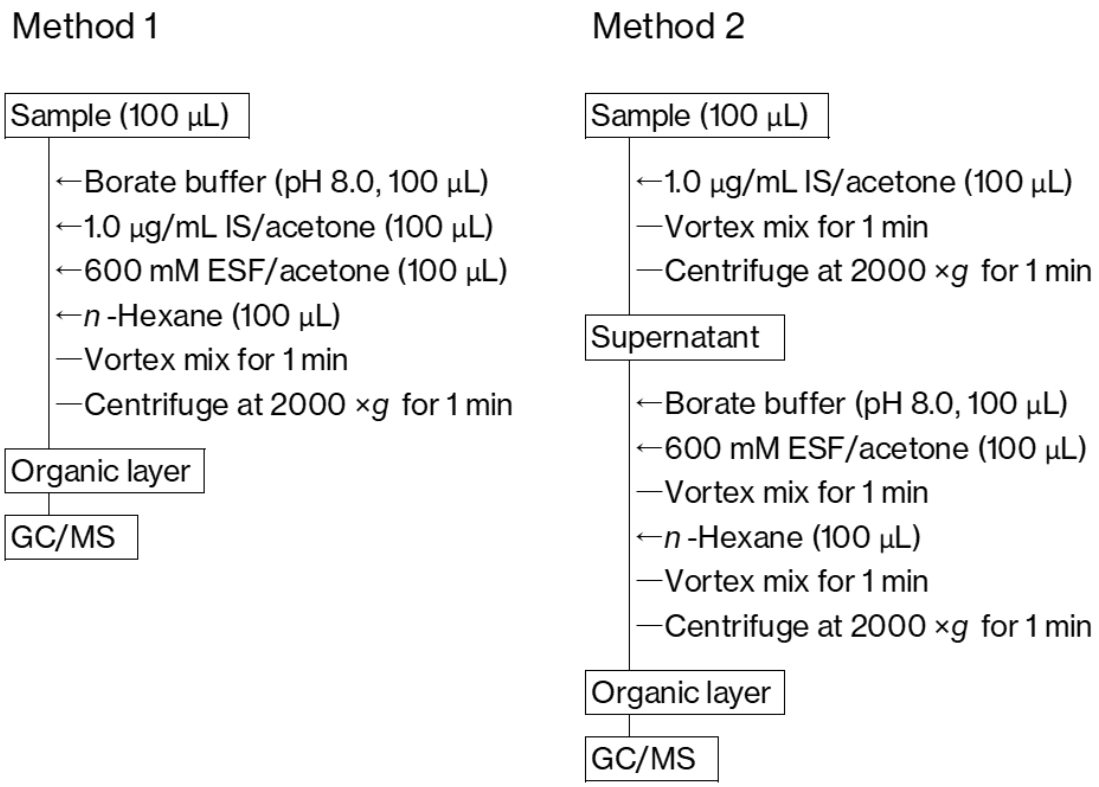


Fig. 3.4 Comparison of derivatization methods 1 and 2 for sulfide ion analysis in whole blood.

In method 2, we performed protein precipitation to remove excess proteins, hypothesizing that this would allow for more efficient derivatization. However, as shown in Fig. 3.5, the efficacy of derivatization was much lower in method 2, which may be due to the distribution of sulfide ion in the precipitated proteins.

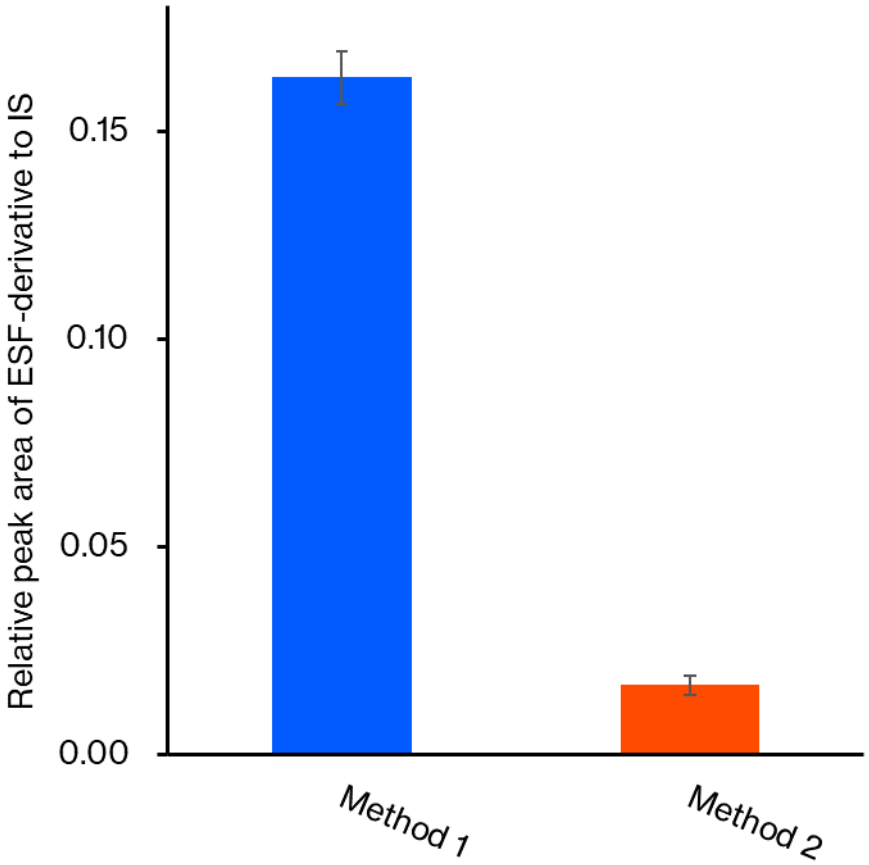


Fig. 3.5 Results of a comparison of derivatization methods for the analysis of sulfide ion in whole blood. Each operation was performed in three times and the mean value is presented with SD.

3.3.4 Effect of Protein Precipitation Solvents

Fig. 3.6 shows the effect of protein precipitation solvent. Acetone provided the highest concentration of S-ESF$_2$ although its efficacy of the protein removal efficacy is not reported to be the highest among the three examined organic solvent (the protein removal efficacy: methanol < acetone < acetonitrile).[29] Thus, a high removal efficacy of acetonitrile may result in coprecipitation of sulfide ion with proteins, leading to the low efficacy of derivatization.

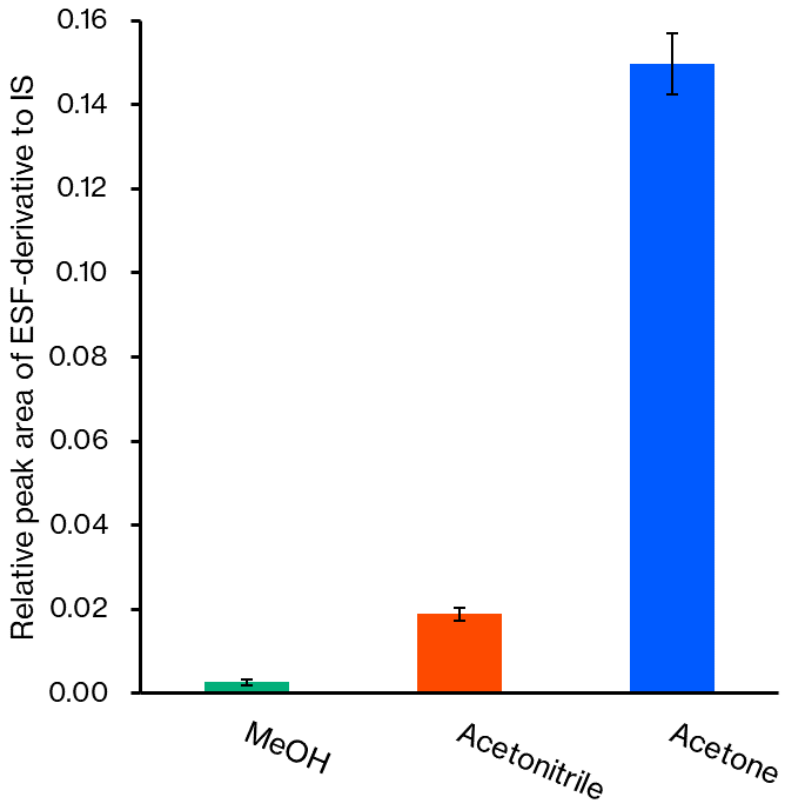


Fig. 3.6 Effect of different protein precipitation solvents for the derivatized samples. Each operation was performed three times and the mean value is presented with SD.

3.3.5 Derivatization Times

Fig. 3.7 shows the effect of reaction time of derivatization on the recovered amount of S-ESF_2 derivative. The amount of S-ESF_2 was almost plateau at 1 min, proving the rapid reaction of S-ESF_2 formation.

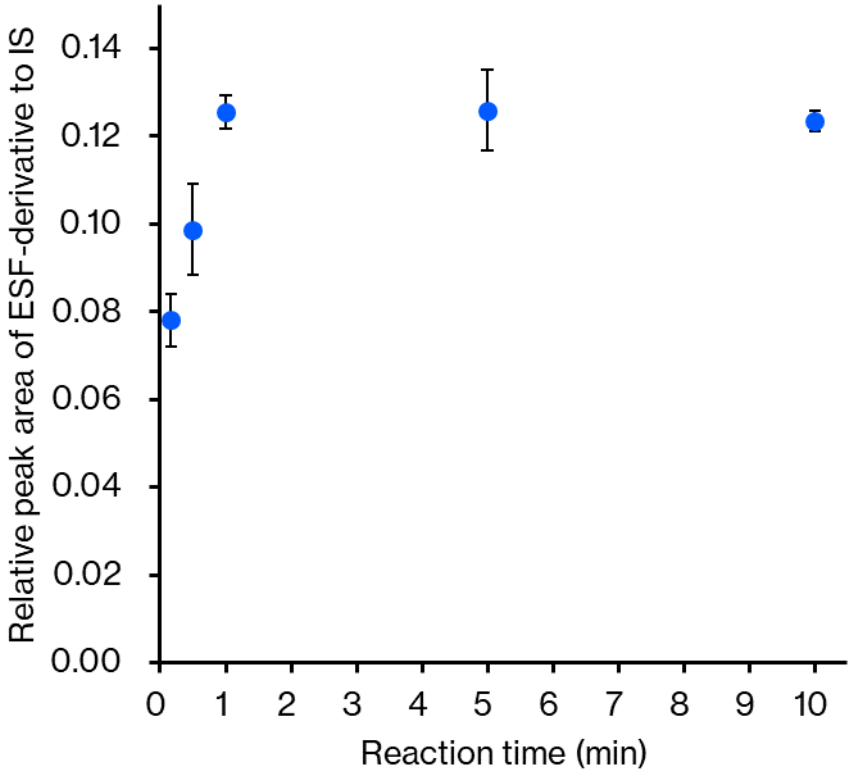


Fig. 3.7 Effect of the reaction time on sulfide ion derivatization. Each experiment was performed three times, and the mean value is presented with SD.

3.3.6 Effect of Derivatization pHs

Fig. 3.8 shows the effect of the buffer pH on the derivatization of sulfide ion with ESF. Because yield of S-ESF_2 was found to be highest at pH 8, we set pH 8 as the optimal pH for the derivatization. The lower yields at the lower pH (pH < 8) will result from the low reactivity in derivatization due to the protonation of sulfide ion at low pH (pKa_1 of H_2S is 6.9).[30] The lower yields at higher pH (pH > 8) may result from reaction of ESF with lysine residues of proteins in the blood, which will interfere with the derivatization of sulfide ion.

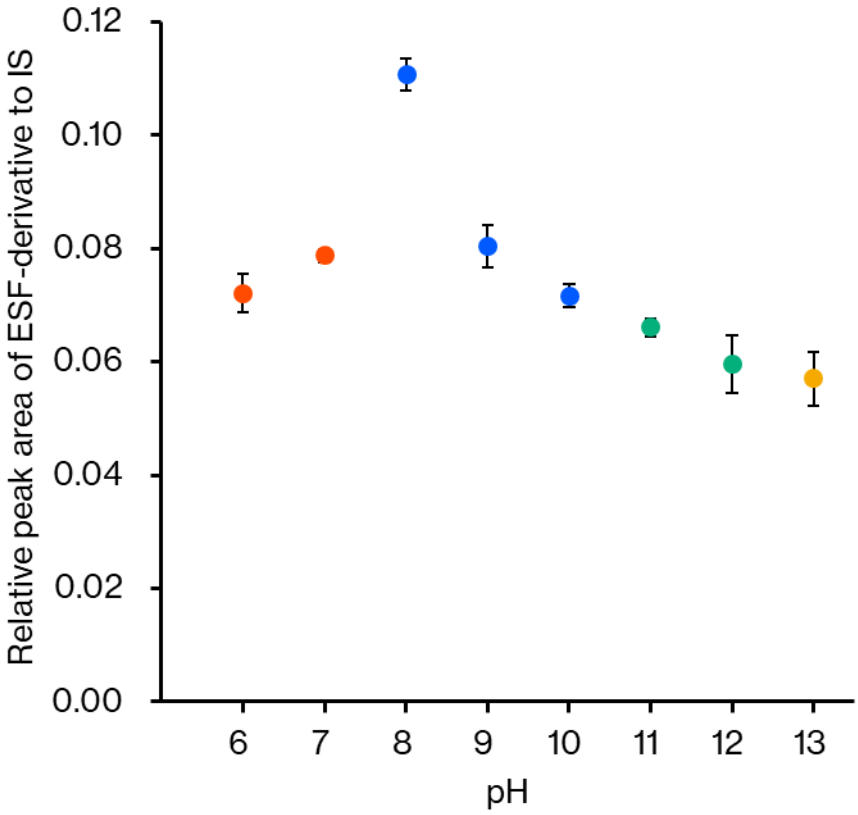


Fig. 3.8 Effect of the buffer solution pH on derivatization. Each operation was performed three times and the mean value is presented with SD. The results for different buffers are shown using the following colors: KH_2PO_4/NaOH (red), H_3BO_3/KCl/NaOH (blue), Na_2HPO_4/NaOH (green), and KCl/NaOH (yellow).

3.3.7 Effect of Extraction Solvents

Fig. 3.9 shows the effect of extraction solvents on the recovery of S-ESF$_2$. *n*-Hexane showed the highest extraction efficiency, indicating that a non-polar solvent is more suitable for the extraction of S-ESF$_2$.

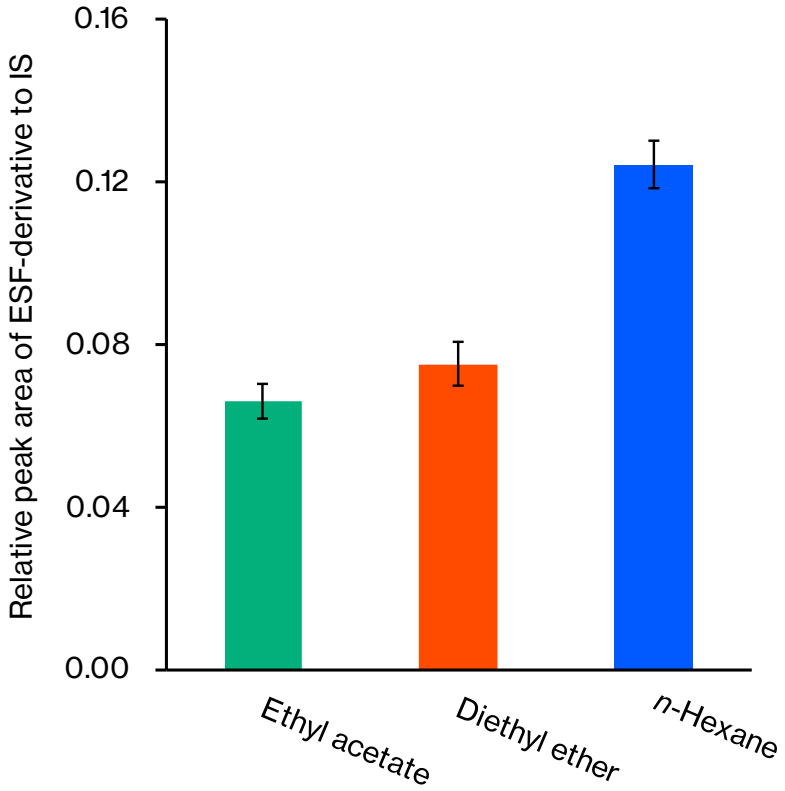


Fig. 3.9 Effect of different extraction solvents for the derivatized samples. Each operation was performed three times and the mean value is presented with SD.

3.4 Analytical Ability

A calibration curve was prepared using solutions with sulfide ion concentrations ranging from 0.05 to 10.0 μg/mL (0.0015 - 0.31 mM) (Fig. 3.10). The linearity of the calibration curve was evaluated based on the relationship between the signal intensity of S-ESF_2 and the sulfide ion concentration. The area ratio of the base peak of S-ESF_2 at *m/z* 86 to the base peak of the IS at *m/z* 188 was plotted against the sulfide ion concentration. Linear regression analysis using the weighting factor of *1/x* showed good linearity (R^2 = 0.9999).

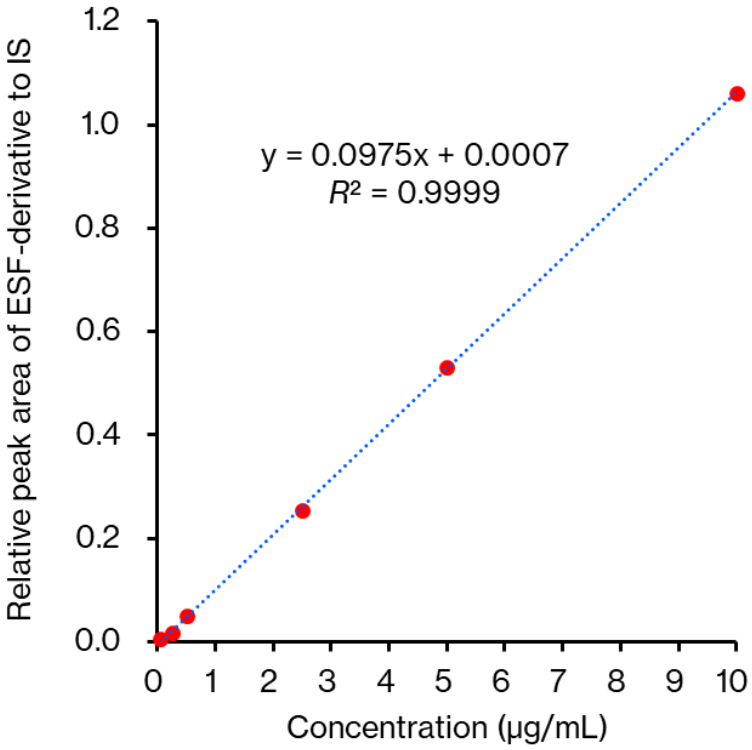


Fig. 3.10 Calibration curve showing the peak areas for S-ESF_2 generated from sulfide ion concentrations ranging from 0.05 to 10.0 μg/mL.

To assess the precision and accuracy of the method, we analyzed whole blood samples adjusted to four concentrations (0.05, 0.10, 1.0, and 8.0 μg/mL) five times each, and repeated the analysis for three days (Table 3.1). The limit of detection based on a S/N ratio of 3 was 0.01 μg/mL, and the limit of quantification based on a S/N ratio of 10 was 0.05 μg/mL. The results showed that the method was very stable and highly reproducible.

Table 3.1 Intra- and inter-day precision and accuracy.

Nominal concentration (μg/mL)	Intra-day (n = 5)			Inter-day (n = 3)		
	Mean ± SD	Precision (% RSD) [a]	Accuracy (% bias) [b]	Mean ± SD	Precision (% RSD) [a]	Accuracy (% bias) [b]
0.05	0.05 ± 0.001	1.9	3.1	0.05 ± 0.001	2.1	2.1
0.10	0.10 ± 0.003	3.4	-2.8	0.10 ± 0.003	3.2	1.6
1.00	0.99 ± 0.034	3.4	-1.1	0.99 ± 0.005	0.5	-1.5
8.00	8.30 ± 0.119	1.4	3.7	8.08 ± 0.174	2.2	1.0

[a] Precision (% relative standard deviation) was defined as (standard deviation/mean peak area ratio to the IS) × 100.

[b] Accuracy (% bias) was defined as [(measured concentration − nominal concentration)/nominal concentration] × 100.

3.5 Comparison with Conventional Methods

3.5.1 HS-GC/MS

The analytical ability of our method was compared with HS-GC/MS, which directly detects H_2S. The whole blood samples spiked with four concentrations of sulfide ion were analyzed three times by each method (Table 3.2). The analysis using HS-GC/MS was conducted following the reported protocol.[25] Briefly, 500 μL of whole blood sample containing sulfide ion and 500 μL of an aqueous solution containing 0.01 vol% of methanol as IS were mixed in a 20-mL glass vial. To this solution, 1 mL of acetone was added, followed by vortex for 5 sec. Then, 500 μL of phosphoric acid was added, the vial was quickly sealed, and another 5 sec of vortex was performed. Subsequently, the sulfide ion in the sample was analyzed by using HS-GC/MS.

Table 3.2 summarized the analytical results of each method. An excellent correlation coefficient was obtained between the two methods (R^2 = 0.9995). Since HS-GC/MS directly detects hydrogen sulfide, it lacks distinctive mass spectrum features (Fig. 3.11), leading to low specificity. In contrast, our method derivatizes sulfide ion, enabling their identification using characteristic fragment ions (Fig. 3.2 B). This method fulfills the requirements for using forensic samples as legal evidence.

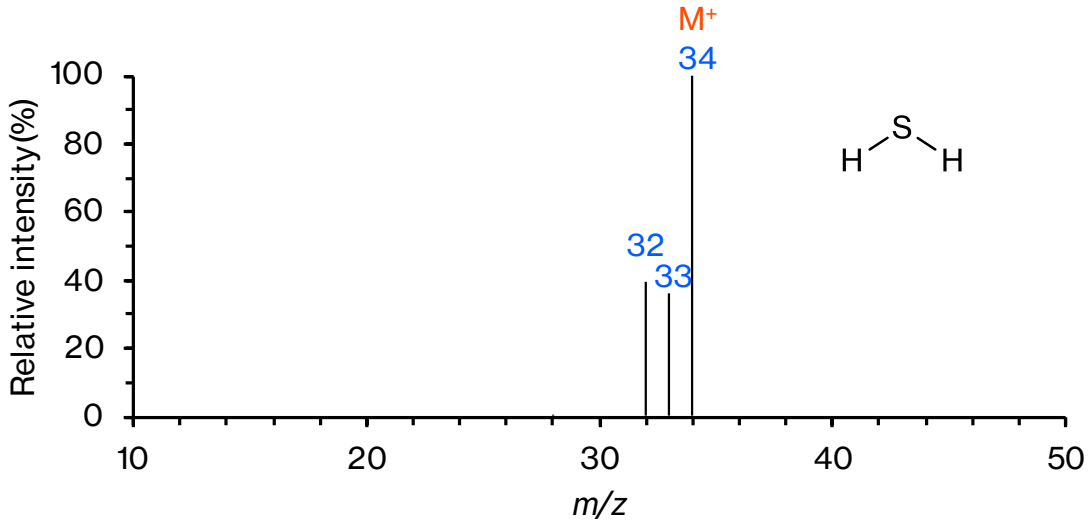


Fig. 3.11 Mass spectra of H_2S. The molecular ion peak at *m/z* 34 was observed for H_2S.

Table 3.2 Results of sulfide ion quantification in simulated samples.

Nominal concentration (μg/mL)	HS-GCMS [a]		Proposed method	
	Mean ± SD	Precision (% RSD) [b]	Mean ± SD	Precision (% RSD) [b]
0.50	0.54 ± 0.066	12.1	0.52 ± 0.010	1.9
1.00	1.03 ± 0.133	12.9	1.01 ± 0.018	1.8
5.00	5.25 ± 0.210	4.0	5.29 ± 0.092	1.7
8.00	8.29 ± 0.411	5.0	8.09 ± 0.104	1.3

[a] Values measured by the established HS-GC/MS method.[25]

[b] Precision (% relative standard deviation) was defined as (standard deviation/mean peak area ratio to the IS) × 100 (n = 3).

3.5.2 PFB Derivatization GC/MS

In the analysis of sulfide ion in blood, we compared the conventional method with PFBBr as the derivatization reagent, to the proposed method. It has been pointed out that the derivatization GC/MS method using PFBBr has issues with the precision of quantitative values. Therefore, we compared the relative standard deviation (RSD) at the same concentration for both methods and conducted validation. The analysis using PFB derivatization was conducted following the reported protocol.[4] Briefly, 0.8 mL of 5 mmol/L TDMBA chloride aqueous solution, 0.5 mL of 20 mmol/L PFBBr in ethyl acetate, and 2.0 mL of 1.0 μmol/L 1,3,5-TBB in ethyl acetate were added as IS. To this solution, 200 μL of blood sample containing sulfide ion was added and then vortexed for 1 min. Then, 0.10 g of potassium dihydrogen phosphate was added and the solution was vortexed for 10 sec. After centrifuging at 2000 × *g* for 1 min, the organic layer was transferred to a separate glass tube and 1.0 μL of it was analyzed by GC/MS (Fig. 3.12).

As shown in Table 3.3, the RSD became larger for the PFB derivatization method, especially near the lower limit of quantification. The PFB derivatization method requires the addition of a surfactant to solubilize hydrophobic PFB, which may increase the deviation of yield of derivatization reaction. On the other hand, our method allows derivatization without surfactants, which may enable more reproducible derivatization reaction.

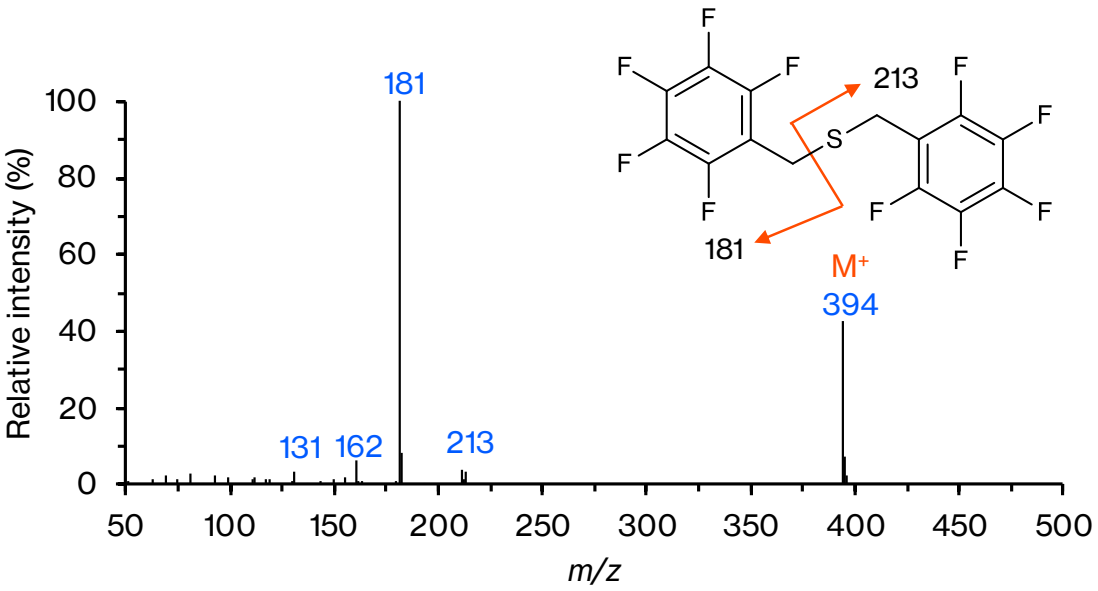


Fig. 3.12 Mass spectra of S-PFB$_2$. The molecular ion peak at *m/z* 394 was observed for S-PFB$_2$.

Table 3.3 Comparison of statistical variability with PFB derivatization methods.

Nominal concentration (μg/mL)	PFB derivatization [a]		Proposed method	
	Mean ± SD	Precision (% RSD) [b]	Mean ± SD	Precision (% RSD) [b]
0.05	0.05 ± 0.008	31.0	0.05 ± 0.001	1.9
0.10	0.08 ± 0.012	20.5	0.10 ± 0.003	3.4
1.00	0.80 ± 0.053	6.9	0.99 ± 0.034	3.4
8.00	8.89 ± 0.443	5.0	8.30 ± 0.119	1.4

[a] Values measured by the established PFB derivatization method.[4]

[b] Precision (% relative standard deviation) was defined as (standard deviation/mean peak area ratio to the IS) × 100 (n = 5).

3.6 Summary

We have proposed ESF as a derivatization reagent of sulfide ion for the highly specific GC/MS analysis of sulfide ion in whole blood based on the characteristic fragment ions. ESF derivatized sulfide ion rapidly and selectively without interference from the abundant proteins in whole blood. Compared to a conventional derivatization method using PFBBr, ESF enabled more reproducible derivatization. The high specificity and reproducibility will make our method a valuable technique not only in forensic science but also in various fields of sulfide analysis.

3.7 References

1. Ma X., Zheng G., Liang M., Xie D., Martinelli G., Sajjad W., Xu W., Fan Q., Li L., Du L., Zhao Y., Occurrence and Origin of H_2S from Volcanic Reservoirs in Niudong Area of the Santanghu Basin, NW China, *Geofluids*, **2019**, 1-10, 2019.
2. Zuo Z., Chang J., Lu Z., Wang M., Lin Y., Zheng M., Zhu D.Z., Yu T., Huang X., Liu Y., Hydrogen sulfide generation and emission in urban sanitary sewer in China: what factor plays the critical role?, *Environmental Sci.: Water Research & Technol.*, **5**, 839-848, 2019.
3. Brglez S., Risk assessment of toxic hydrogen sulfide concentrations on swine farms, *J. Cleaner Production*, 312, 2021.
4. Kage S., Ikeda H., Ikeda N., Tsujita A., Kudo K., Fatal hydrogen sulfide poisoning at a dye works, *Legal Medicine*, **6**, 182-186, 2004.
5. Tangerman A., Measurement and biological significance of the volatile sulfur compounds hydrogen sulfide, methanethiol and dimethyl sulfide in various biological matrices, *J. Chromatogr. B*, **877**, 3366-3377, 2009.
6. Szabo C., A timeline of hydrogen sulfide (H_2S) research: From environmental toxin to biological mediator, *Biochem. Pharmacol*., **149**, 5-19, 2018.
7. Kang Q., Zhu Z., Liu Z., Li F., He Y., Yang Y., Lei S., Yuan Z., Zhu X., A novel hydrogen sulfide donor reduces neuroinflammation and seizures by activating ATP-sensitive potassium channels, *Neuroscience Research*, 2023.
8. Shi X., Li H., Guo F., Li D., Xu F., Novel ray of hope for diabetic wound healing: Hydrogen sulfide and its releasing agents, *J. Advanced Research*, 2023

9. Moretti M., Ballardini M., Siodambro C., Tronconi L., Osculati A.M.M., Freni F., Vignali C., Morini L., Fatal poisoning of four workers in a farm: Distribution of hydrogen sulfide and thiosulfate in 10 different biological matrices, *Forensic Sci. Int.*, **316**, 110525, 2020.

10. Maebashi K., Iwadate K., Sakai K., Takatsu A., Fukui K., Aoyagi M., Ochiai E., Nagai T., Toxicological analysis of 17 autopsy cases of hydrogen sulfide poisoning resulting from the inhalation of intentionally generated hydrogen sulfide gas, *Forensic Sci. Int.*,**207**, 91-95, 2011.

11. Logan M., Baxter C., Chemical suicides: Hazards and how to manage them, *J. Chem. Health & Safety*, **25**, 2-5, 2018.

12. Beauchamp R.O., James S.B., James A.P., Craig J.B., Dragana A.A., A Critical Review of the Literature on Hydrogen Sulfide Toxicity, *CRC Critical Reviews in Toxicol.*, **13**, 25-97, 1984.

13. Whitfield N.L., Kreimier E.L., Verdial F.C., Skovgaard N., Olson K.R., Reappraisal of H_2S/sulfide concentration in vertebrate blood and its potential significance in ischemic preconditioning and vascular signaling, *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, **294**, R1930-1937, 2008.

14. Jarosz A.P., Yep T., Mutus B., Microplate-based colorimetric detection of free hydrogen sulfide, *Anal. Chem.*, **85**, 3638-3643, 2013.

15. Ahn Y.J., Han S.H., Lee G.J., Rapid and simple colorimetric detection of hydrogen sulfide using an etching-resistant effect on silver nanoprisms, *Mikrochim. Acta*, **188**, 129, 2021.

16. Su L., Liu B., Wu N., Li J., Zhang T., Colorimetric detection of hydrogen sulfide based on novel magnetic functional composites, *Biosensors and Bioelectronics: X 11*, **11**, 100155, 2022.

17. Rath H.J., Wimmer J., Gas chromatographic analysis of traces of hydrogen sulfide in hydrogen, *Chromatographia*,**13**, 513-514, 1980.

18. Kage S., Nagata T., Kimura K., Kudo K., Extractive alkylation and gas chromatographic analysis of sulfide, *J. Forensic Sci.*, **33**, 217-222, 1988.

19. Vitvitsky V., Banerjee R., H_2S analysis in biological samples using gas chromatography with sulfur chemiluminescence detection, *Methods Enzymol*, **554**, 111-123, 2015.

20. Thompson R., Perry J. D., Stanforth S. P., Dean J. R., Rapid detection of hydrogen sulfide produced by pathogenic bacteria in focused growth media using SHS-MCC-GC-IMS, *Microchem. J.*, **140**, 232-240, 2018.

21. Zha L., Duan W., Wen D., Guo Y., Yan J., Chang Y., Cai J., Ding Y., Rapid Determination of H_2S Poisoning in a Forensic Study Using a Novel Fluorescence Assay Based on Zn/Cu@BSA Nanoclusters, *Aust. J. Chem.*, **71**, 142-148, 2018.

22. Chen S., Hou P., Wang J., Fu S., Liu L., A rapid and selective fluorescent probe with a large Stokes shift for the detection of hydrogen sulfide, *Spectrochim Acta A Mol Biomol Spectrosc*, **203**, 258-262, 2018.

23. Yu Y., Li G., Wu D., Zheng F., Zhang X., Liu J., Hu N., Wang H., Wu Y., Determination of Hydrogen Sulfide in Wines Based on Chemical-Derivatization-Triggered Aggregation-Induced Emission by High-Performance Liquid Chromatography with Fluorescence Detection, *J. Agric. Food. Chem.*, **68**, 876-883, 2020.

24. Hyspler R, Tichá A, Indrová M, Zadák Z, Hysplerová L, Gasparic J, Churácek J., A simple, optimized method for the determination of sulphide in whole blood by GC–MS as a marker of bowel fermentation processes, *J. Chromatogr. B*, **770**, 255-259, 2002.

25. Iwamuro Y., Murakami T., Ishimaru R., Sakamoto Y., Chinaka S., Determination of hydrogen sulfide in whole blood by headspace-gas chromatography/mass spectrometry, *Jap. J. Forensic Sci. and Technol.*, **25**, 151-158, 2020.

26. Japanese Industrial Standards Committee, Methods for determination of hydrogen sulfide in flue gas, JIS K0108, https://www.jisc.go.jp/index.html.

27. McAnalley B.H., Lowry W.T., Oliver R.D., Garriott J. C., Determination of Inorganic Sulfide and Cyanide in Blood Using Specific Ion Electrodes: Application to the Investigation of Hydrogen Sulfide and Cyanide Poisoning, *J. Anal. Toxicol.*, **3**, 111-114, 1979.

28. Peter E.A., Shen X., Shah S.H., Pardue S., Glawe J.D., Zhang W.W., Reddy P., Akkus N.I., Varma J., Kevil C.G., Plasma free H_2S levels are elevated in patients with cardiovascular disease, *J. Am. Heart Assoc.*, **2**, e000387, 2013.

29. Blanchard J., Evaluation of the relative efficacy of various techniques for deproteinizing plasma samples prior to high-performance liquid chromatographic analysis, *J. Chromatogr.*, **226**, 455-460, 1981.

30. Hershey J.P., Plese T., Millero F.J., The pK1* for the dissociation of H_2S in various ionic media, Geochim. *Cosmochim. Acta*, **52**, 2047-2051, 1988.

CHAPTER 4

Identification of Cresol Isomers in Whole Blood: Development of a GC/MS Using Ethenesulfonyl Fluoride as a Derivatization Reagent

4.1 Introduction

Cresol is a compound in which one methyl group is substituted at the ortho, meta, or para position (*o*-, *m*-, or *p*-) of the hydroxyl group of phenol. It is a water-soluble, yellow-brown viscous liquid with a distinctive odor.[1,2] Cresol is produced in industrial processes such as the refining of petroleum and the dry distillation of coal, and is used as a disinfectant, preservative, and raw material for functional materials.[3,4] Cresol has a strong bactericidal effect and is commonly available as a preservative, disinfectant, and maggot killer.[2] There have been cases of death due to poisoning by accidental ingestion of these products.[5] The concentration of unconjugated cresol in the blood during cresol poisoning is 9-74 μg/mL in survival cases and 90-957 μg/mL in fatal cases.[6-9] Furthermore, the isomer content of cresol varies depending on the product.[6,10] In recent years, *p*-cresol has been reported to be effective in the prevention and treatment of diabetes and has been administered,[11] and it is also known that *p*-cresol is produced in the human body through the metabolism of tyrosine.[12,13] In addition, *o*-cresol is produced by the metabolism of toluene contained in products such as thinners,[14,15] so cresol can be detected in the human body even outside of poisoning cases. Therefore, in the field of forensic chemistry, it is extremely important and essential to identify the different isomers of cresol to distinguish between different cresol products, to make judgments on poisoning cases, and to prevent

misidentification of the cause of death.

As for the analysis methods of cresol, there have been reports on spectrophotometric methods,[16] GC,[17-19] and LC.[20,21] However, these methods lack specificity for cresol as they are based on absorbance or retention time. As a mass spectrometry method that can specifically detect cresol, derivatization GC/MS methods such as silylation and PFB derivatization have been developed,[13-15] but silylation does not proceed if water is present in the system, and PFB derivatization has drawbacks such as tear-inducing properties of the derivatization agent, difficult handling of reagents, long reaction times, and low reproducibility.[22]

Therefore, this chapter examined ESF as a derivatization reagent for the analysis of cresol in whole blood. When this ESF derivatization analysis method was applied to the analysis of cresol, a phenolic *O*-nucleophile, from whole blood, it was confirmed that ESF rapidly reacts with cresol in whole blood and produces cresol-ESF derivatives suitable for GC separation and mass spectrometric detection (Fig. 4.1).

This chapter attempted to develop a method for the identification of isomers of cresol in whole blood using ESF, a reagent that does not cause tearing and allows derivatization reactions in water, enabling rapid and simple derivatization.

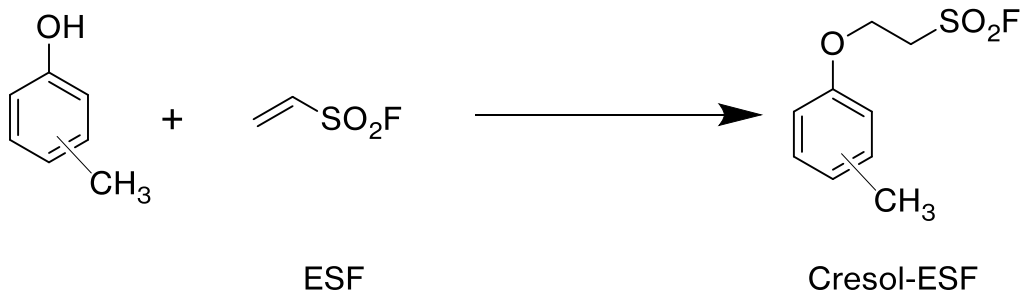


Fig. 4.1 The derivatization reaction of cresol by ethenesulfonyl fluoride (ESF).

4.2 Experimental

4.2.1 Materials

o-Cresol (FUJIFILM Wako Pure Chemical Corp.), *m*-Cresol (FUJIFILM Wako Pure Chemical Corp.), and *p*-Cresol (FUJIFILM Wako Pure Chemical Corp.) were each diluted with water to prepare 1000 μg/mL aqueous solutions as their respective standard solutions. These standard solutions were further diluted to various concentrations with water and added to human whole blood (Cosmo Bio Co., Ltd.) to prepare the samples. The derivatization reagent was prepared by dilution of ESF (Tokyo Chemical Industry Co., Ltd.) to a concentration of 5% by volume with acetonitrile. Phenanthrene-d_{10} (FUJIFILM Wako Pure Chemical Corp.) was used as the IS and dissolved in acetonitrile to a concentration of 10.0 μg/mL. Phenanthrene-*d*10 was selected as a substance with high stability and non-reactivity toward ESF in the IS selection process. The purified water used in this study was obtained from a Milli-Q Integral 5 system (Merck Millipore).

4.2.2 Derivatization and Extraction Procedure

100 μL of the cresol-containing whole blood sample was placed in a 1.5-mL capacity poly container, to which 100 μL of a 10 μg/mL IS acetonitrile solution was added. The mixture was vortexed for 1 min and then centrifuged at 2000 × *g*. 100 μL of the supernatant was transferred to another poly container, and 100 μL of borate buffer (pH 10) and 100 μL of a 5 vol% ESF acetonitrile solution were added. This was vortexed for 30 sec. 100 μL of ethyl acetate was added to the reaction solution, which was then vortexed for 1 min and centrifuged at 2000 × *g*. The resulting organic layer was analyzed using GC/MS.

4.2.3 GC/MS Analysis

GC/MS determination was performed on a GCMS-QP2020 NX system (Shimadzu) equipped with a DB-5MS capillary column (30 m × 0.25 mm *i.d.*, 0.25 μm film thickness, Agilent Technologies). The GC oven temperature was held at 60°C for 2 min and increased to 230°C at 10°C/min (held for 1 min). The injection mode was splitless and the carrier gas was helium at a flow rate of 1.6 mL/min. The MS interface temperature was 250°C and the ion source temperature was 250°C. The system was operated with a filament current of 150 μA, an electron energy of 70 eV, and in EI mode. The derivative was identified in full scan mode, and data were acquired from *m/z* 30 to 300. Quantitation was performed in SIM mode with monitoring of ions at *m/z* 107 and 218 for cresol-ESF and *m/z* 188 and 160 for the IS (the quantitation ions are underlined).

4.3 Results and Discussion

4.3.1 Optimization of Cresol Derivatization by ESF

First, it was checked whether cresol reacts with ESF in water. The results confirmed that all isomers of cresol react with ESF, forming cresol-ESF derivatives.

Next, it was verified whether cresol reacts with ESF in whole blood. 100 μL of a cresol-containing sample was placed in a 1.5-mL capacity poly container, to which 100 μL of a 10 μg/mL IS acetonitrile solution was added. The mixture was vortexed for 1 min and then centrifuged at 2000 × *g*. Afterward, 100 μL of the supernatant was transferred to another poly container. To this, 100 μL of borate buffer (pH 10) and 100 μL of a 5 vol% ESF acetonitrile solution (600 mM) were added, and then the mixture was vortexed for 30 sec. Then, 100 μL of ethyl acetate was added to the reaction solution, which was vortexed for 1 min and centrifuged at 2000 × *g*. The results showed that cresol rapidly reacts with ESF in whole blood, forming cresol-ESF derivatives.

The extracted ion chromatogram and mass spectrum of the obtained cresol-ESF derivatives are shown in Fig. 4.2. As shown in Fig. 4.2 A, each isomer of cresol-ESF was well separated on the DB-5MS column. Additionally, each isomer of cresol-ESF exhibited characteristic mass spectra, with molecular ion peaks (*m/z* 218) and ions derived from cresol (*m/z* 107) regenerated after the loss of -$CH_2CH_2SO_2F$ (Fig. 4.2 B-D).

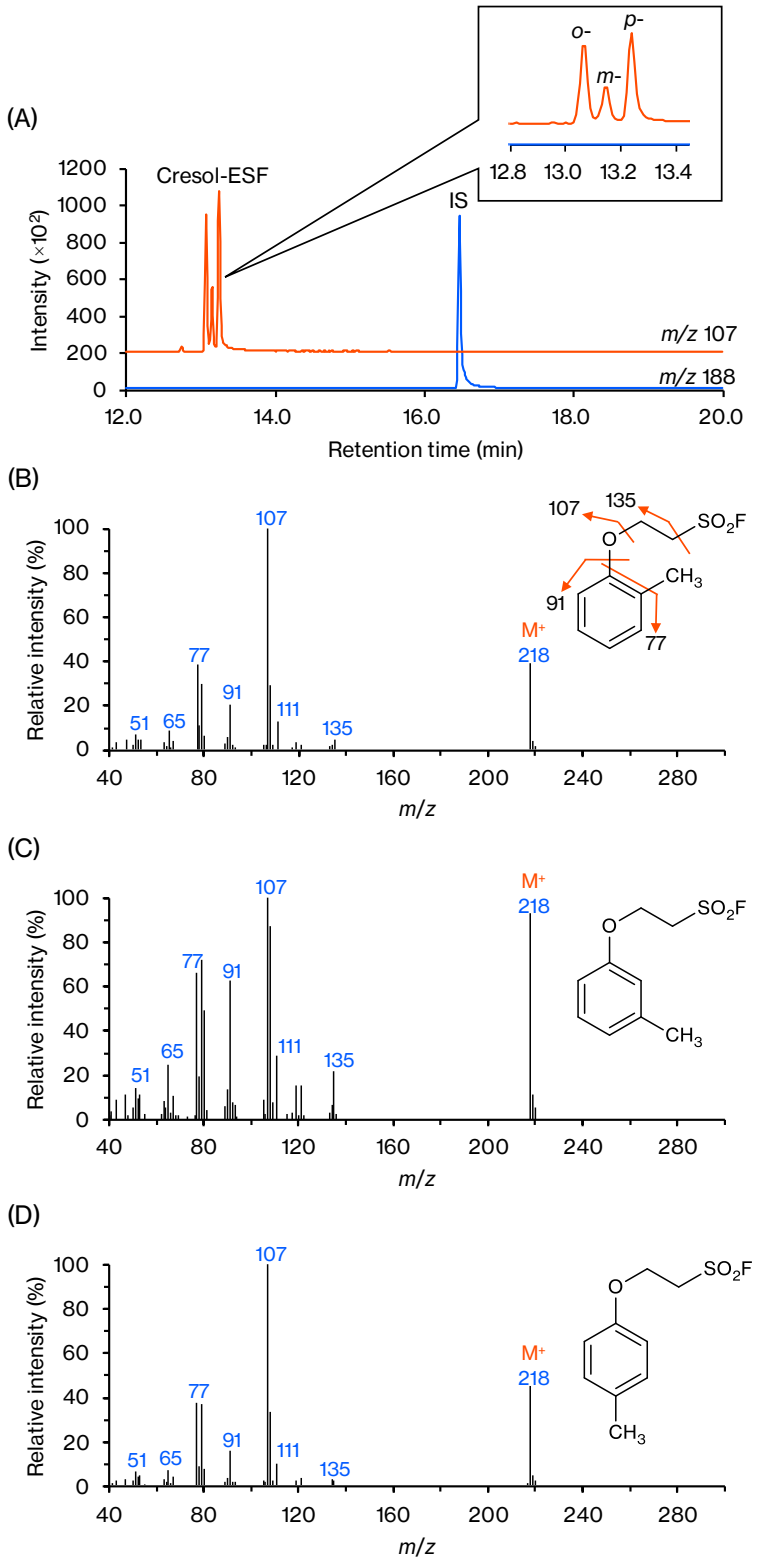


Fig. 4.2 Extracted ion chromatograms of the ESF-derivatized cresol (A). Mass spectra of cresol-ESF: *o*-cresol-ESF (B), *m*-cresol-ESF (C), and *p*-cresol-ESF (D).

4.3.2 Stability

After extracting the cresol-ESF derivatives with ethyl acetate, the vial was left at room temperature on an autosampler, and GC/MS analysis was conducted continuously every 4 hours for 48 hours (Fig. 4.3).

As a result, no changes in peak areas were observed for any of the isomers, and no decomposition within the measurement sample solution was detected. Furthermore, % RSD for the peak area values of the cresol-ESF derivatives over 48 hours was below 5% for all isomers. These findings indicate that all isomers of the cresol-ESF derivatives possess high stability.

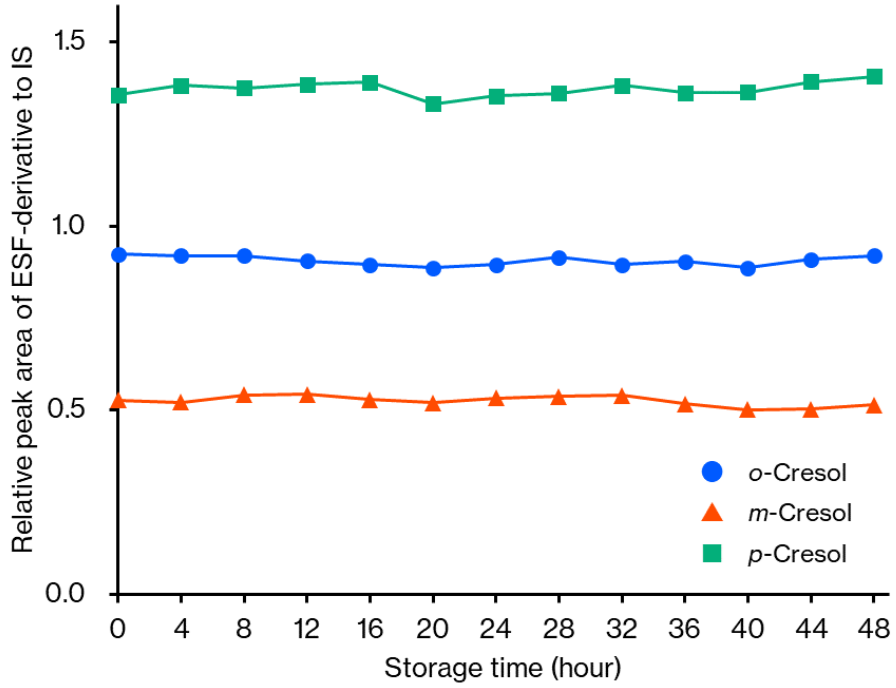


Fig. 4.3 Effect of storage time of the ethyl acetate extract of the derivatized reaction solution on the GC/MS analysis. The extract was stored at room temperature before the analysis.

4.3.3 Derivatization Timings

The timing of the derivatization was investigated, either after protein precipitation of the whole blood (Method 1) or simultaneous with protein precipitation (Method 2), as shown in Fig. 4.4.

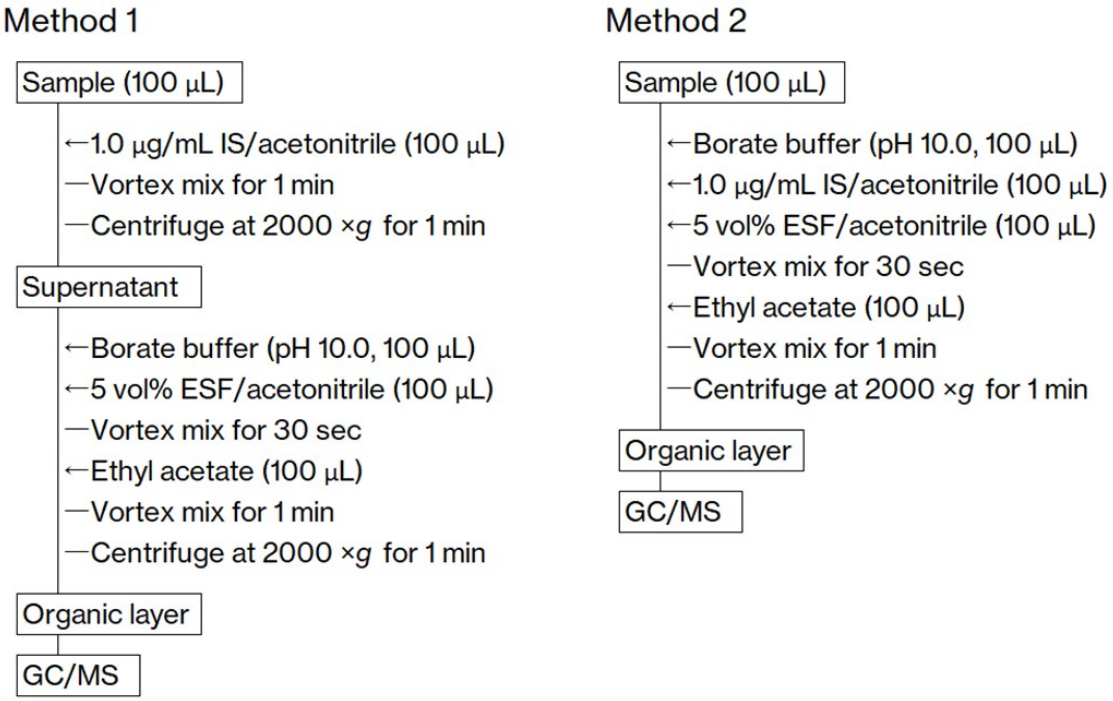


Fig. 4.4 Comparison of derivatization methods for cresol analysis in whole blood.

When the derivatization was performed after protein precipitation, cresol was well derivatized. In contrast, when derivatization was carried out simultaneously with protein precipitation, little to no progression of derivatization was observed (Fig. 4.5). This is thought to be due to the proteins in the whole blood reacting with ESF and consuming it, thereby interfering with the reaction between cresol and ESF.

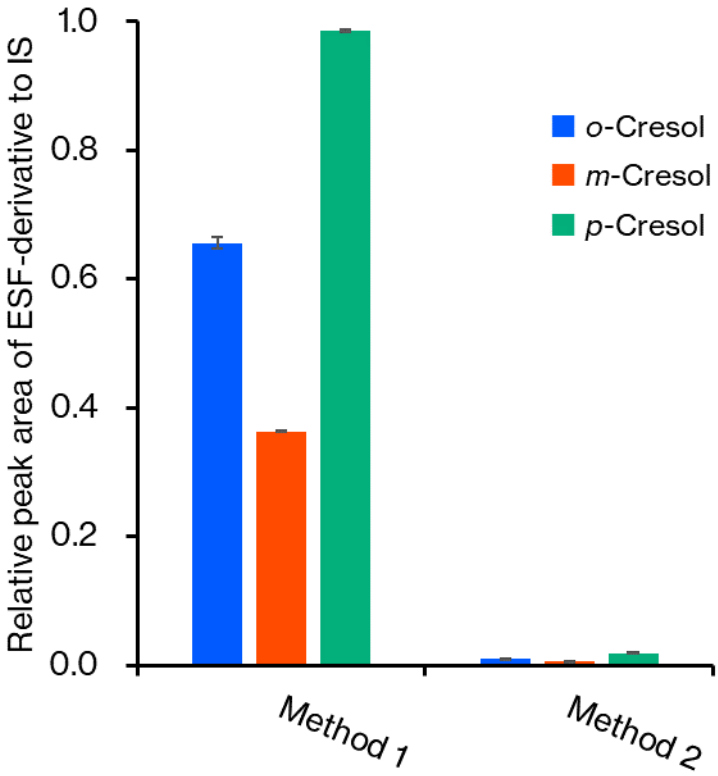


Fig. 4.5 Results of a comparison of derivatization methods for the analysis of cresol in whole blood. Each operation was performed three times and the mean value is presented with SD.

4.3.4 Effect of Protein Precipitation Solvents

The use of methanol, acetone, and acetonitrile as solvents for protein precipitation of whole blood and for ESF was investigated (Fig. 4.6).

Generally, the efficiency of protein precipitation is reported to be methanol < acetone < acetonitrile.[23] In this study, while protein precipitation and derivatization of cresol were possible with all solvents, the use of acetonitrile showed the highest efficiency of derivatization for all isomers. This is likely because acetonitrile has the highest efficiency in protein precipitation, thus most effectively removing blood proteins that could interfere with the reaction of cresol with ESF. Therefore, acetonitrile was selected as the solvent for both protein precipitation and the reaction process.

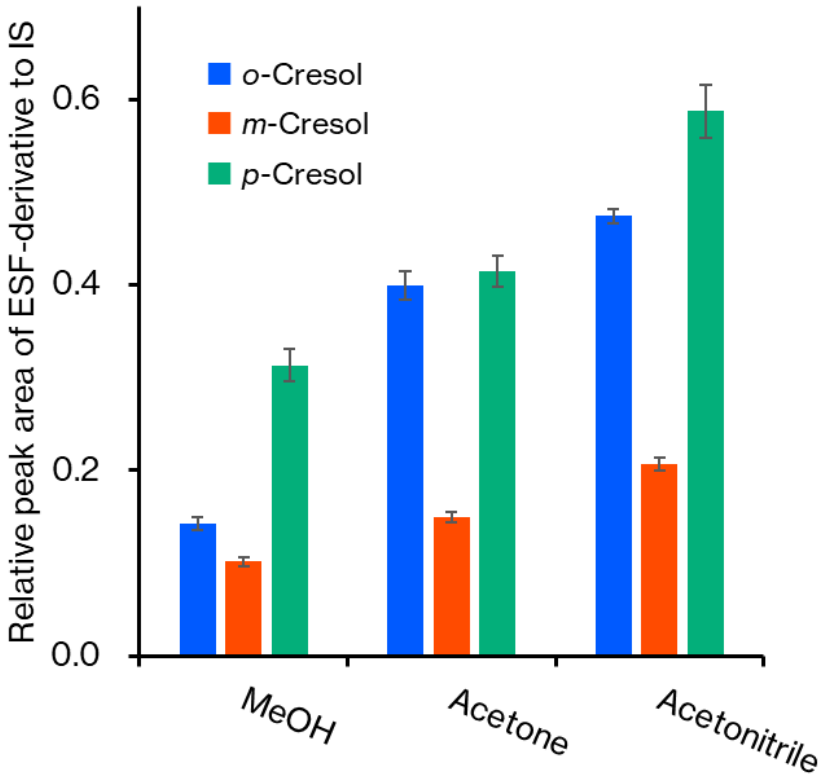


Fig. 4.6 Effect of different protein precipitation solvents for the derivatized samples. Each operation was performed three times and the mean value is presented with SD.

4.3.5 Derivatization Times

To optimize the reaction time for the derivatization of cresol with ESF, the effect of stirring times of 0, 10, 30, 60, and 90 sec was investigated (Fig. 4.7).

The results showed that cresol reacts with ESF immediately after addition, and the amount of derivatized product increased with the stirring time, reaching a maximum at 30 sec. No further increase in the amount of cresol-ESF derivative was observed with longer stirring times. Therefore, the reaction time for the cresol and ESF reaction was set at 30 sec.

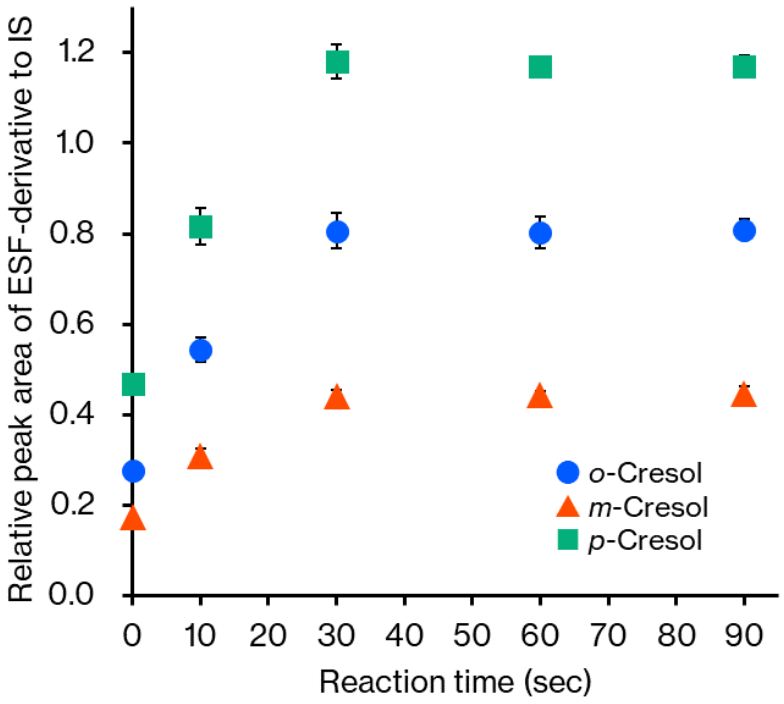


Fig. 4.7 Effect of the reaction time on cresol derivatization. Each operation was performed three times and the mean value is presented with SD.

4.3.6 Effect of Derivatization pHs

The optimal pH for the derivatization of cresol with ESF was investigated (Fig. 4.8). Buffers used for pH adjustment were potassium dihydrogen phosphate (pH 6 and 7), boric acid (pH 8, 9, and 10), and disodium hydrogen phosphate (pH 11).

The results showed that cresol barely reacted with ESF under acidic and neutral conditions. This is thought to be because the p*K*a of cresol is 10.3,[24] and at pH values below p*K*a - 1, most of the cresol remains unionized and lacks nucleophilicity, preventing the Michael reaction from proceeding. Conversely, at pH 11 and above, the N-terminus of blood proteins is neutralized, becoming nucleophiles, leading to the reaction of proteins with ESF and consequent consumption of ESF, which interferes with the reaction between cresol and ESF. Based on these results, pH 10 was determined to be the optimal pH.

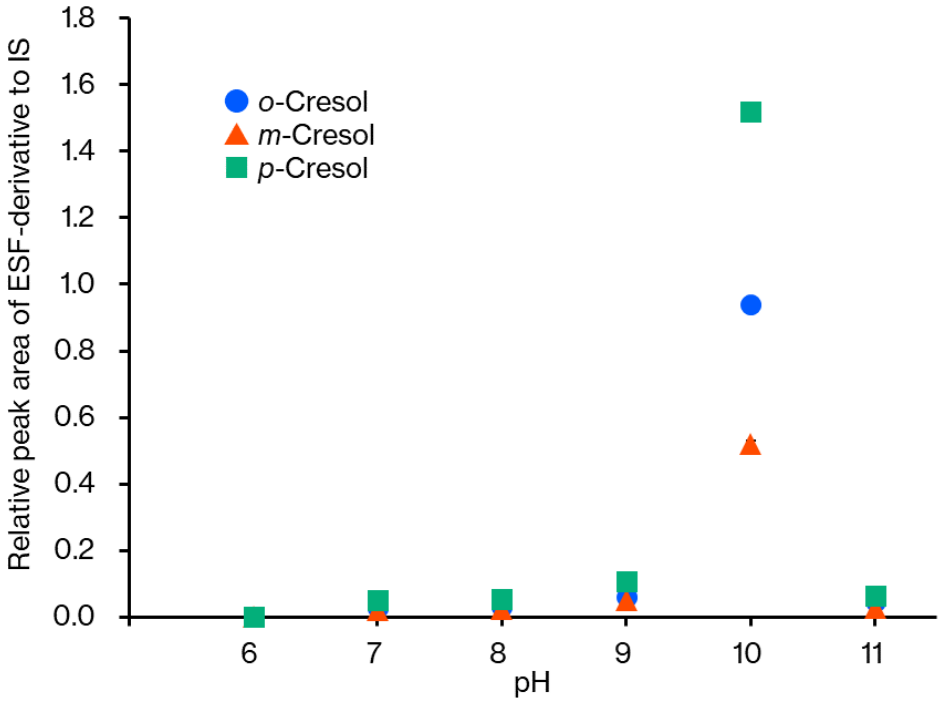


Fig. 4.8 Effect of the buffer solution pH on derivatization. Each operation was performed three times and the mean value is presented with SD.

4.3.7 Effect of Extraction Solvents

The extraction solvents *n*-hexane, diethyl ether, and ethyl acetate were evaluated for their effectiveness in extracting derivatives after derivatization (Fig. 4.9).

All the solvents used in this study were capable of extracting the cresol-ESF derivatives. However, it was found that the use of ethyl acetate resulted in the highest extraction efficiency of the ESF derivatives. The cresol-ESF derivative becomes a relatively polar molecule due to the introduction of the sulfonyl fluoride group, and among the extraction solvents tested, ethyl acetate, being the most polar, exhibited the highest extraction efficiency.

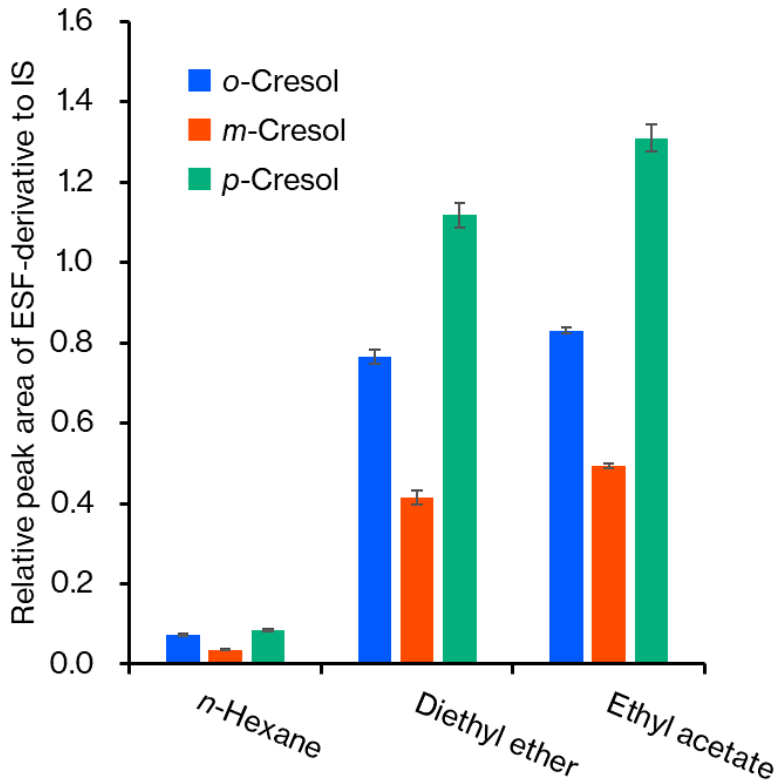


Fig. 4.9 Effect of different extraction solvents for the derivatized samples. Each operation was performed three times and the mean value is presented with SD.

4.4 Analytical Ability

Quantitative analysis of cresol in whole blood was performed using the optimized conditions. The calibration curves for each isomer of cresol are shown in Fig. 4.10. Cresol-ESF derivatives exhibited good linearity in the range of 0.50 – 100 μg/mL, with a correlation coefficient of R^2 = 0.9999, calculated with *1/x* weighting. This concentration range was suitable for both the toxic and lethal concentrations of cresol.

In addition, the accuracy and precision of the method were evaluated at four concentrations (0.50, 1.0, 10.0, 75.0 μg/mL) for intra-day (n = 5) and inter-day (n = 3) (Table 4.1). As shown in Table 4.1, the accuracy and precision for all cresol-ESF derivatives were within ±10% for both intra-day and inter-day. Furthermore, the limit of detection was 0.10 μg/mL (S/N ratio > 3), and the limit of quantification was 0.50 μg/mL (S/N ratio > 10).

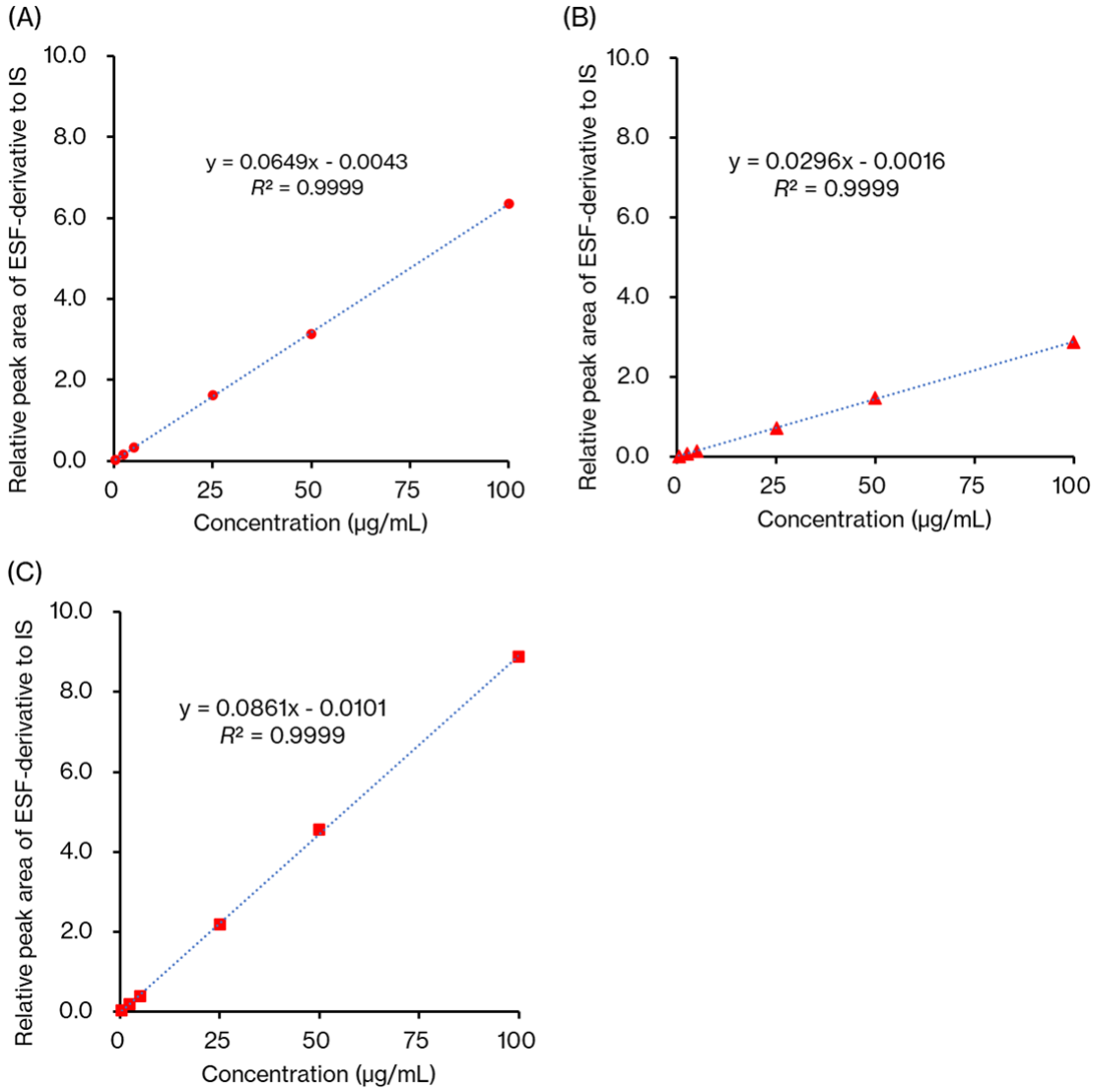


Fig. 4.10 Calibration curve showing the peak areas for cresol-ESF generated from cresol isomers concentrations ranging from 0.5 to 100.0 µg/mL. The results for cresol isomers are represented in the following panels: *o*-cresol (A), *m*-cresol (B), and *p*-cresol (C).

Table 4.1 Intra- and inter-day precision and accuracy.

Compounds	Nominal concentration (μg/mL)	Intraday (n = 5)			Interday (n = 3)		
		Mean ± SD	Precision (% RSD) [a]	Accuracy (% bias) [b]	Mean ± SD	Precision (% RSD) [a]	Accuracy (% bias) [b]
o-Cresol	0.50	0.49 ± 0.01	2.4	-2.3	0.51 ± 0.02	2.3	-1.2
	1.0	1.02 ± 0.02	2.1	1.9	0.99 ± 0.03	4.2	-3.3
	10.0	9.73 ± 0.15	1.5	-2.7	9.47 ± 0.51	5.4	-5.2
	75.0	74.2 ± 2.16	2.9	-1.1	74.7 ± 1.44	1.9	-0.1
m-Cresol	0.50	0.49 ± 0.02	3.6	-2.6	0.52 ± 0.03	4.9	4.3
	1.0	1.01 ± 0.02	4.7	1.8	1.03 ± 0.01	1.1	2.6
	10.0	10.5 ± 0.19	1.8	5.1	10.0 ± 0.43	4.3	0.3
	75.0	77.1 ± 1.48	1.9	2.8	77.4 ± 1.73	2.2	3.3
p-Cresol	0.50	0.51 ± 0.01	2.6	3.0	0.51 ± 0.02	4.9	1.2
	1.0	1.00 ± 0.02	2.4	0.4	1.00 ± 0.01	0.2	0.3
	10.0	9.71 ± 0.26	2.7	-2.9	9.67 ± 0.41	4.2	-3.3
	75.0	71.6 ± 2.84	4.0	-4.5	73.6 ± 2.79	3.8	-1.8

[a] Precision (% relative standard deviation) was defined as (standard deviation/mean peak area ratio to the IS) × 100.

[b] Accuracy (% bias) was defined as [(measured concentration − nominal concentration)/nominal concentration] × 100.

4.5 Summary

A selective, rapid, and simple method for the detection, quantification, and identification of all isomers of cresol in whole blood using GC/MS was developed. ESF was effective in efficiently derivatizing each isomer of cresol in whole blood. With ESF, cresol in whole blood could be rapidly, efficiently, and selectively derivatized without interference from proteins and other impurities in the blood, and the cresol-ESF derivatives were easily separable on GC. Furthermore, the cresol-ESF derivatives were stable in ethyl acetate extract at room temperature for 48 hours. In addition, when quantitative analysis of each isomer of cresol in whole blood was performed, good linearity was observed in the range of 0.50 to 100 μg/mL. This concentration range covers the blood concentrations found in cases of poisoning and fatalities due to cresol. The accuracy and precision for intra-day and inter-day were evaluated at four concentrations: limit of quantification, low, medium, and high levels, and all were found to be within ± 10%, indicating that this quantitative method is highly reliable.

This method is a novel analytical approach that enables the detection, quantification, and identification of all isomers of cresol in whole blood with a rapid and simple pretreatment process. The process involves using ESF as the derivatizing agent, adding borate buffer (pH 10) and the derivatizing reagent to the acetonitrile protein precipitation supernatant of whole blood, stirring for 30 sec, and then extracting with ethyl acetate.

4.6 References

1. Organization World Health, Environmental Health Criteria 168, available from https://www.inchem.org/documents/ehc/ehc/ehc168.htm
2. Morinaga Y., Fuke C., Arao T., Miyazaki T., Quantitative analysis of cresol and its metabolites in biological materials and distribution in rats after oral administration. *Leg. Med. (Tokyo)*, **6**, 32-40, 2004.
3. Ramalho M. B., Duraes A. F. S., Silverio F. O., Pinho G. P., Determination of three cresol isomers in sewage sludge by solid-liquid extraction with low temperature purification and gas chromatography-mass spectrometry. *J. Environ. Sci. Health. B*, **55**, 184-192, 2020.
4. Nikunj B, Anjali P., Liquidphase tert-butylation of cresols catalysed by 12-tungstophosphoricacid and 12-tungstosilicicacid supported onto neutral alumina. *Catalysis Letters*, **113**, 99-103, 2007.
5. Doughmi D., Bennis L., Berrada A., Derkaoui A., Shimi A., Khatouf M., Severe ARDS Complicating an Acute Intentional Cresol Poisoning. *Case Reports in Critical Care*, **2019**, 6756352, 2019.
6. Monma-Ohtaki J., Maeno Y., Nagao M., Iwasa M., Koyama H., Isobe I., Seko-Nakamura Y., Tsuchimochi T., Matsumoto T., An autopsy case of poisoning by massive absorption of cresol a short time before death. *Forensic Sci. Int.*, **126**, 77-81, 2002.
7. Yashiki M., Kojima T., Miyazaki T., Chikasue F., Ohtani M., Gas chromatographic determination of cresols in the biological fluids of a non-fatal case of cresol intoxication. *Forensic Sci. Int.*, **47**, 21-29, 1990.
8. Bruce A.M., Cresol poisoning. *Med. Sci. Law*, **16**, 171-176, 1976.

9. Arthurs G.J., Wise C.C., Coles G.A., Poisoning by cresol. *Anaesthesia*, **32**, 642-643, 1977.

10. Boatto G., Nieddu M., Carta A., Pau A., Lorenzoni S., Manconi P., Serra D., Determination of phenol and *o*-cresol by GC/MS in a fatal poisoning case. *Forensic Sci. Int.*, **139**, 191-194, 2004.

11. Brial F., Alzaid F., Sonomura K., Kamatani Y., Meneyrol K., Le Lay A., Pean N., Hedjazi L., Sato T. A., Venteclef N., Magnan C., Lathrop M., Dumas M. E., Matsuda F., Zalloua P., Gauguier D., The natural metabolite 4-cresol improves glucose homeostasis and enhances beta-cell function. *Cell Rep.*, **30**, 2306-2320 e2305, 2020.

12. Smith E.A., Macfarlane G.T., Dissimilatory amino acid metabolism in human colonic bacteria. *Anaerobe*, **3**, 327-337, 1997.

13. Ikematsu N., Kashiwagi M., Hara K., Waters B., Matsusue A., Takayama M., Kubo S. I., Diagnostic meaning of blood *p*-cresol concentration in forensic autopsy cases. *Leg. Med.*, **34**, 27-35, 2018.

14. Marais A. A., Laurens J. B., Analysis of urinary biomarkers for exposure to alkyl benzenes by isotope dilution gas chromatography-mass spectrometry. *J. Sep. Sci.*, **28**, 2526-2533, 2005.

15. Schettgen T., Alt A., Dewes P., Kraus T., Simple and sensitive GC/MS-method for the quantification of urinary phenol, *o*- and *m*-cresol and ethylphenols as biomarkers of exposure to industrial solvents. *J. Chromatogr. B*, **995-996**, 93-100, 2015.

16. López C.M., Loreto L., Rubio Soledad R., Dolores P.B., Simultaneous spectrophotometric determination of *o*-cresol and *m*-cresol in urine by use of the kinetic wavelength-pair method. *Analyst*, **121**, 1647-1652, 1996.

17. Lee X.P., Kumazawa T., Saito K., Takano M., Hattori H., Seno H., Ishii A., Watanabe-Suzuki K., Suzuki O., Sato K., Determination of cresol isomers and phenol in human body fluids by capillary gas chromatography with cryogenic oven trapping. *Anal. Lett.*, **35**, 2093-2103, 2002.

18. Li J., Xu Y., Du W., Jin L., Ren P., Ren F., Xie J.C., Comparative analysis of aroma compounds in Chinese traditional dry-rendered fat by HS/GC-IMS, SPME/GC-MS, and SPME/GC-O. *J. Food Compos.Anal.*, **107**, 2022.

19. Lee H.B., Peart T.E., Carron J.M., Gas chromatographic and mass spectrometric determination of some resin and fatty acids in pulpmill effluents as their pentafluorobenzyl ester derivatives. *J. Chromatogr. A*, **498**, 367-379, 1990.

20. Brega A., Prandini P., Amaglio C., Pafumi E., Determination of phenol, *m*-, *o*- and *p*-cresol, *p*-aminophenol and *p*-nitrophenol in urine by high-performance liquid chromatography. *J. Chromatogr.*, **535**, 311-316, 1990.

21. Wei X., Gilevska T., Wetzig F., Dorer C., Richnow H. H., Vogt C., Characterization of phenol and cresol biodegradation by compound-specific stable isotope analysis. *Environ. Pollut.*, **210**, 166-173, 2016.

22. Hyspler R., Tichá A., Indrová M., Zadák Z., Hysplerová L., Gasparic J., Churácek J., A simple, optimized method for the determination of sulphide in whole blood by GC–MS as a marker of bowel fermentation processes. *J. Chromatogr. B*, **770**, 255-259, 2002.

23. Blanchard J., Evaluation of the relative efficacy of various techniques for deproteinizing plasma samples prior to high-performance liquid chromatographic analysis. *J. Chromatogr.*, **226**, 455-460, 1981.

24. Pearce P.J., Simkins R.J.J., Acid strengths of some substituted picric acids. *Can. J. Chem.*, **46**, 241-248, 1968

CHAPTER 5

Conclusions

5.1 General Conclusion

In the field of forensic science, mass spectrometry is used as a highly sensitive and reliable method for the identification of a substance. The results of this analysis are important evidence at trial and are indispensable in the examination of facts, and its effectiveness has been widely recognized. In recent years, with the introduction of the jury trial system, the importance of objective evidence that is easily understood by the general public has increased. However, due to the wide variety of chemical substances, established analytical methods based on mass spectrometry may not yet exist for some substances. In addition, rapid analytical methods may not be available or may have low accuracy.

In Chapter 2, we reported the development of a rapid and specific analytical method for ammonia in aqueous solutions using GC/MS. Ammonia, which is commonly available as a commercial product for insect bites and stings, is readily available and therefore often involved in incidents such as foreign substance contamination and odor nuisance. Traditional analytical methods have been used primarily to measure ammonia concentrations in air or environmental waters. Existing mass spectrometric methods were limited to the analysis of gaseous ammonia and did not allow direct derivatization and analysis of ammonia in water. Therefore, in this chapter, we developed a new method using ESF, a derivatizing agent capable of reacting with ammonia in water, to enable qualitative and quantitative analysis of ammonia in water using GC/MS. It was confirmed

that ESF can readily react with ammonia, an *N*-nucleophile, in water, facilitating its derivatization into a compound that can be analyzed by GC/MS. Furthermore, by optimizing the reaction temperature, time, and pH, we established a rapid and specific analytical method for ammonia in aqueous solutions.

In Chapter 3, we established a new method for the rapid and simple analysis of sulfide ion in whole blood using GC/MS. Sulfide ion are chemicals often analyzed as intoxicants. The conventional derivatized GC/MS method using PFBBr has been shown to have problems with reagent handling and reproducibility. In addition, the HS-GC/MS method lacks specificity because the mass spectrum obtained is not characteristic. To overcome these problems, ESF, which was shown to be derivatizable in water in Chapter 2, was used as a derivatizing agent for the analysis of sulfide ion in whole blood. In this method, ESF was reacted with sulfide ion during deproteinization, taking into account the load on the mass spectrometer; S-ESF_2 showed a characteristic mass spectrum and the calibration curve also showed good linearity. A comparison with the conventional method was also made. First, this method was compared with HS-GC/MS, and the results showed that this method was able to obtain a characteristic mass spectrum, which proved the high specificity of the method. Second, the PFB derivatization method was compared with the derivatization method using PFBBr as the derivatization reagent; the PFB derivatization method could not perform stable analysis at the lower limit of quantification. On the other hand, this method was able to perform stable analysis even at the lower limit of quantitation. These results demonstrate the high reproducibility of this method. These results established a new method for the rapid, simple, specific, and highly reproducible analysis of sulfide ion in whole blood.

In Chapter 4, we developed a new method for the rapid and simple separation of isomers of cresol in whole blood using GC/MS. Cresol, which is widely used as a disinfectant and preservative due to its strong bactericidal properties, is readily available and therefore implicated in accidental poisoning cases. Identification of the isomers of cresol in biological samples is important in poisoning cases because their content varies. Recently, *p*-cresol has been used in the treatment of diabetes and is known to be produced in the human body by the metabolism of tyrosine. In addition, the ingestion of toluene, found in products such as paint thinner, can lead to the formation of *o*-cresol as a metabolite. Traditional methods for cresol analysis were based on absorbance or retention time and lacked specificity. Mass spectrometric methods have been developed, but they do not perform well in the presence of water. In this study, we used ESF as a derivatizing reagent, which was shown to be effective for sulfide ion analysis in whole blood in Chapter 3. After protein precipitation from whole blood samples, ESF was added to the supernatant to derivatize cresol, allowing good separation of isomers by GC/MS. Quantitative analysis of each cresol isomer in whole blood showed good linearity. Thus, a rapid and simple method for the identification of cresol isomers in whole blood was established.

5.2 Perspective

As technology advances, crimes and accidents are expected to become more complex and sophisticated, and with the increase in new chemical substances, traditional analytical methods may struggle to cope with forensic samples. The derivatization method using ESF demonstrated in this study is particularly useful for the analysis of anionic substances and applies to high matrix samples such as whole blood. The use of ESF may allow the analysis of substances that are difficult to handle with traditional methods, making it a potential first-choice method in drug analysis. The development of rapid substance identification methods can respond to unforeseen situations. In addition, new chemical substances are emerging in increasingly sophisticated drug crimes. Therefore, it is important to continue developing new analytical methods so that chemical substances that could not be handled in the past or new chemical substances can be analyzed by incorporating the latest analytical techniques.

The analytical methods developed in this study are expected to be applied not only in the field of forensic science, but also in other areas where mass spectrometry is used as a confirmatory analytical method, such as clinical analysis, food analysis, and environmental analysis.

Acknowledgments

This doctoral dissertation was completed during my tenure in the Doctoral Program in Systems Life Sciences at Kyushu University, under the guidance of the Katayama Laboratory.

First and foremost, I am deeply grateful to Professor Yoshiki Katayama of the Graduate School of Engineering, Kyushu University, for his consistent, detailed guidance and encouragement throughout this research.

I would like to express my deep appreciation to Associate Professor Takeshi Mori of the Graduate School of Engineering, Kyushu University, for his invaluable teaching and advice in conducting this research, writing academic papers, and during the doctoral thesis defense.

I would like to thank Professor Susumu Kudo of the Graduate School of Systems Life Sciences, Kyushu University, and Professor Noritada Kaji of the Graduate School of Engineering, Kyushu University, for their helpful advice during the doctoral thesis defense.

I am sincerely grateful to Supervisor Akinaga Gohda and Assistant Section Head Kengo Wakigawa of the Fukuoka Prefectural Police Headquarters Forensic Science Laboratory for their support in creating a conducive research environment and for their advice in writing this thesis. I also greatly appreciate the unwavering support of my colleague, Mr. Shin Ogawa, who was always willing to discuss research and thesis writing.

Finally, I would like to express my deepest gratitude to my wife, Rie, for graciously allowing me to enroll in the doctoral program as a working adult and for her unwavering support in my research endeavors. My thanks also go to my son, Natsuki, for his smile even in the times of my research struggles. I am grateful for my family's

encouragement and support in many ways.

This research would not have been possible without their deep understanding, support, and encouragement during these three years.

Ryosuke Shiraki

Kyushu University

January 2024