Determination of Antimicrobial Agents in Non-Formalin Adhesives for Wallpaper

Harunobu Nakashima,* Ichiro Matsunaga, Naoko Miyano, and Mikiya Kitagawa

Osaka Prefectural Institute of Public Health, 3–69 Nakamichi 1-chome, Higashinariku,Osaka, 537–0025, Japan

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To reduce formaldehyde in indoor environments, non-formalin products for wallpaper adhesives have been increased in number. In this study, an analysis method using gas chromatography (GC) and gas chromatography mass spectrometry (GC/MS) specific to each of several antimicrobial agents (2-methyl-4-isothiazolin-3-one (MIT), 5-chloro-2-methyl-4-isothiazolin-3-one (Cl-MIT), 1,2-benzisothiazolin-3-one (BIT), 2-*n*-octyl-4-isothiazolin-3-one (OIT) and 2,4,5,6-tetrachloro-isophthalonitrile (TPN)) was developed aiming to clarify the actual states of utilization of these agents. These antimicrobials have been reported to induce health impairments such as contact dermatitis. Furthermore, seven non-formalin adhesives for wallpaper sold as building materials were analyzed according to the established method. The present results demonstrated that these adhesives contained a combination of antimicrobials (MIT, Cl-MIT, OIT and TPN), and one of them contained TPN at a high concentration (2,480 μg/g), which is highly contact-allergenic.

Key words —— isothiazolones, tetrachloroisophthalonitrile, gas chromatographic determination, non-formalin adhesive, allergic contact dermatitis, indoor environmental pollution

INTRODUCTION

Health impairments such as multiple chemical sensitivities and sick-house syndrome caused by the chemical substances in household articles and building materials are becoming important social problems.[1–4] In particular, indoor environmental pollution due to formalin is a serious problem at present, and many attempts to remove the compound from indoor have been progressing. Therefore, non-formalin adhesives for wallpaper that have safety and ecological features as selling points are now increasing. However, the type and content of antimicrobial agents used as the substitutes for formalin are not known in most of those adhesives. To clarify the actual states of utilization of these antimicrobials, we attempted to develop an analytical method for each of the following antimicrobial agents usable for wallpaper which have been reported to have caused health impairments such as contact dermatitis.

Four isothiazolones, including 2-methyl-4-isothiazolin-3-one (MIT), 5-chloro-2-methyl-4-isothiazolin-3-one (Cl-MIT), 1,2-benzisothiazolin-3-one (BIT), 2-*n*-octyl-4-isothiazolin-3-one (OIT) and 2,4,5,6-tetrachloro-isophthalonitrile (TPN), were chosen as test chemicals. Several adhesive products for building materials were analyzed according to the respectively developed methods. It was thus demonstrated that a combination of four antimicrobial agents (MIT, Cl-MIT, OIT and TPN) were detectable in the respective adhesives, and one of them contained a high concentration of TPN with high contact allergenicity (2,480 μg/g). In this report, the analytical methods and the analytical data are described and the results are discussed from the aspect of toxicity assessment.

*To whom correspondence should be addressed: Osaka Prefectural Institute of Public Health, 3–69 Nakamichi 1-chome, Higashinariku, Osaka 537–0025, Japan. Tel.: +81- 6-6972-1321; Fax: +81-6-6972-2393; E-mail: hrnakaji@iph.pref.osaka.jp

MATERIALS AND METHODS

Samples ——Seven non-formalin adhesive products for wallpaper, sold as building materials, were used as subjects for analysis. Six samples were prepared in the paste state, and only sample No. 3 was a solid powder.

Reagents —— As the standard substances, we used NS-500W containing 3.2% MIT and 10.3% Cl-MIT (Nagase Chemical Co., Ltd., Hyogo, Japan) for MIT and Cl-MIT, Ploxcel PL (100% purity, Zeneca Co., Ltd., Tokyo, Japan) for BIT, Permachem PD containing 4.75% OIT (Permachem Asia Co., Ltd., To-

Table 1. Operating Conditions of GC and GC/MS

FPD-GC conditions
Column: SPB-5
(ϕ 0.53 mm $\times$ 30 m, film thickness 1.5 μm , Supelco)
Column temperature:
100°C (1 min) – 10°C/min – 280°C (20 min)
Injection inlet temperature: 250°C,
Detector temperature: 280°C
Carrier gas flow rate: He 20 ml/min,
Injection volume: 2 μl
ECD-GC conditions
Column: PTE-5
(ϕ 0.25 mm $\times$ 30 m, film thickness 0.25 μm , Supelco)
Column temperature:
60°C (1 min) – (20°C/min) – 285°C (3 min.)
Injection inlet temperature: 200°C,
Detector temperature: 300°C
Carrier gas flow rate
(Injection inlet pressure): He 110 kPa
Injection volume: 1 μl (splitless)
GC/MS conditions
Column: DB-5MS
(ϕ 0.25 mm $\times$ 30 m, film thickness 0.1 μm, J & W)
Column temperature:
80°C (1 min) – 8°C/min – 280°C (4 min)
Injection inlet temperature: 250°C,
Interface temperature: 280°C
Carrier gas flow rate: He 1 ml/min.,
Injection volume: 2 μl (splitless)
Ionization voltage: 70 eV (EI method)

kyo, Japan) for OIT, and the standard product (100% purity, Sannopuco, Tokyo, Japan) for TPN. A graphite carbon black cartridge column was ENVI-CARB (1 g/12 ml, Supelco Co., Ltd., Bellefonte, U.S.A.), and the silica cartridge column was Sep-Pak Plus (Waters Co., Ltd., Massachusetts, U.S.A.). Organic solvents such as *n*-hexane were used for residual pesticide analysis.

GC and GC/MS Operating Conditions ——FPD-GC (5890 Series II, Yokogawa Hewlett Packard Co., Ltd., Tokyo, Japan) and ECD-GC (GC-17A, Shimadzu Co., Ltd., Kyoto, Japan) were used for the quantitative analyses of isothiazolones and TPN, respectively. GC/MS (5890 Series II, 5970, Yokogawa Hewlett Packard) was used for identification and confirmation of each compound detected in the samples.

Table 1 shows the operating conditions of GC and GC/MS.

Preparation of Test Samples

MIT and Cl-MIT: One-half gram of an adhesive sample was put into a 50 ml centrifuge tube and shaken for 5 min with 20 ml of purified water for extraction. The mixture was centrifuged for 5 min at 3000 rpm. The aqueous extract was applied onto the graphite carbon cartridge column. After running with 20 ml of purified water, 20 ml of methanol was injected to elute MIT and Cl-MIT. After evaporating the eluate, the residue was dissolved in *n*-hexane and determined by FPD-GC.

BIT: One-half gram of a sample was put into an 80 ml centrifuge tube and shaken for 5 min with 30 ml of purified water and 30 ml of diethyl ether/cyclohexane mixture (4 : 1) for extraction. The mixture was centrifuged for 5 min at 3000 rpm. The supernatant (diethyl ether/cyclohexane phase) was put into a 200 ml round-bottomed flask. A similar operation was repeated twice after the addition of 30 ml of a diethyl ether/cyclohexane mixture. The extract was concentrated to 5 ml and applied onto the silica cartridge column. After running with 20 ml of *n*-hexane, 20 ml of acetone was injected to elute BIT, and the eluate was determined by FPD-GC.

OIT: One-half gram of a sample was put into an 80 ml centrifuge tube and shaken for 5 min with 30 ml of purified water and 30 ml of cyclohexane. The mixture was centrifuged for 5 min at 3000 rpm. The supernatant (cyclohexane phase) was transferred into a round-bottomed flask. A similar operation was repeated twice, with the addition of 30 ml of cyclohexane. The extract was concentrated to 5 ml and applied onto a silica cartridge column. After running with 20 ml of *n*-hexane, 20 ml of *n*-hexane/acetone mixture (1 : 1) was injected to elute OIT, and the eluate was subjected to FPD-GC analysis.

TPN: One-half gram of a sample was put into an 80 ml centrifuge tube and shaken for 5 min with 30 ml of purified water and 30 ml of cyclohexane. The mixture was centrifuged for 5 min at 3000 rpm. The supernatant (cyclohexane phase) of 2 ml was applied onto a silica cartridge column. After running with 40 ml of *n*-hexane, 20 ml of *n*-hexane containing 1% acetone was injected to elute TPN, and the eluate was determined by ECD-GC.

RESULTS AND DISCUSSION

Preparation of Standard Solutions

Since BIT and TPN were almost pure, these products were dissolved in acetone to make the stock solutions, which were diluted with *n*-hexane to prepare working standards. For data as to the content

of MIT and Cl-MIT, we referred to the package leaflet of the product. Thus, these product data were used as the standard values. The product was dissolved with acetone and appropriately diluted with *n*-hexane to prepare the working standards for GC. For the recovery experiment, the stock solution was diluted with purified water. For OIT, no data as to product content or any product information were obtainable. So, the concentration of OIT in Permachem PD was determined first of all. The peak of OIT in Permachem PD was identified by GC/MS and compared with the peak area of undecane, a same carbon number compound, by FID-GC to estimate the concentration of OIT. The concentration of OIT in Permachem PD was 4.75%. Thus, an acetone solution of Permachem PD was prepared and appropriately diluted with *n*-hexane to prepare the working standards.

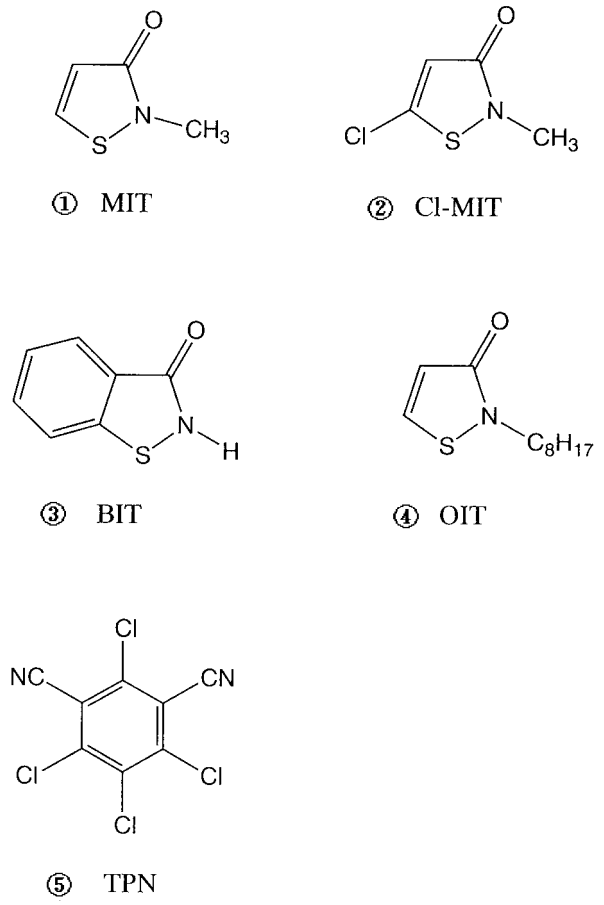

Fig. 1. Chemical Structures of the Respective Antimicrobial Agents

① MIT: 2-methyl-4-isothiazolin-3-one, ② Cl-MIT: 5-chloro-2-methyl-4-isothiazolin-3-one, ③ BIT: 1,2-benzisothiazolin-3-one, ④ OIT: 2-*n*-octyl-4-isothiazolin-3-one, ⑤ TPN: 2,4,5,6-tetrachloroisophthalonitrile.

Examination of Detection Methods

Since all antimicrobial isothiazolones contain sulfur, these compounds were detected by FPD-GC (S filter, 393 nm), and TPN was detected by ECD-GC. Figure 1 shows the chemical structures of those compounds.

FPD-GC Conditions: There is a report on the measurement of MIT and Cl-MIT in some methylisothiazolone formulations (Kathon CG, Euxyl K 100, *etc*.) by HPLC.[5] However, since highly sensitive analysis is needed for the simultaneous measurement of isothiazolones used as antimicrobial agents in adhesives, we investigated the operating conditions of the GC method for such a purpose. Since all antimicrobial isothiazolones contain sulfur, FPD-GC (S filter, 393 nm) was employed for detection. There are few reports on analytical methods for isothiazolones using GC. In particular, BIT was decomposed by adsorption in a GC column, as assumed from its structure, and was less sensitive than other isothiazolones. Therefore, BIT was methylated with diazomethane, and two kinds of derivatives (*O*-methylated and *N*-methylated derivatives) were induced simultaneously. Although detection sensitivity was raised by the use of these derivatives, it became necessary to examine the synthetic ratio of the two derivatives. At the concentration used in these test samples, the compound was detectable without methylation. Therefore, methylation was not conducted in this study because other isothiazolones scarcely submitted to adsorption decomposition in the GC column. As a result of various GC conditions being examined, discrimination of the four antimicrobial agents and their isolation became possible using FPD-GC under the conditions mentioned above. Determination was made using the respective calibration curves ranging from 0.64–6.4 ng (0.32–3.2 μg/ml, detection limit 0.32 ng) for MIT, 1.03–20.6 ng (0.515–10.3 μg/ml, detection limit 0.25 ng) for Cl-MIT, 8–40 ng (4–20 μg/ml, detection limit 8 ng) for BIT, and 0.38–5.7 ng (0.19–2.85 μg/ml, detection limit 0.19 ng) for OIT.

ECD-GC conditions: Although a variety of analytical methods for TPN in agricultural products,[6–9] soil,[10,11] water[12,13] and air[14–16] have been reported, no method for analyzing TPN in adhesives was found. In the present study, highly sensitive ECD-GC was used, and determination was performed under the above conditions using the calibration curve in the range of 5–50 pg (5–50 ng/ml, detection limit 3 pg).

Pretreatment Methods

For each antimicrobial agent, pretreatment methods were examined because the polarity was different among the test chemicals.

Extraction and Cleanup for MIT and Cl-MIT: The adhesives contained starch as a principal ingredient and were water-soluble. Therefore, it was necessary to dissolve a sample in the water in order to extract antimicrobial agents from these adhesives. However, as the solubilities of two antimicrobial agents were higher in water than organic solvents, their extraction from water to an organic solvent was difficult. Thus, solid–phase extraction was examined using an ODS column, polystyrene polymer column and graphite carbon column. The graphite carbon cartridge column (ENVI-CARB 1 g/12 ml, Supeluco Co., Ltd.) was optimum for extracting both agents. An aqueous solution of MIT (3.2 or 32 μg) and Cl-MIT (10.3 or 103 μg) was applied onto the column, then 40 ml of purified water was passed through. At that time, 99% of MIT and 100% of Cl-MIT were retained in the column. Then, 20 ml of methanol was passed through and the both agents were completely eluted from the column. Thus, not only solvent exchange for assay by GC, but also elimination of polar substances became possible. In addition, the elimination of non-polar substances was possible by a liquid–liquid partition using *n*-hexane and water (1 : 1). But this process was omitted, because the samples were all water-soluble and did not seem to contain non-polar substances.

Only sample No. 3 was a solid powder. On the leaflet, it was instructed to add the attached antiseptic into the preparation diluted with 7 or 8 volumes of water. Since no antimicrobial agent was present in the original product, the recovery test was made for each antimicrobial agent using this product. To 0.5 g of Sample No. 3 prepared with water, MIT (3.2 or 32 μg) or Cl-MIT (10.3 or 103 μg) was added, and the recovery test was conducted according to the procedures. The recovery rate was in a range of 95 to 101% (Coefficient of variation CV = 4.0%, $n = 3$).

Extraction and Cleanup for BIT: Solvents to extract BIT from aqueous solution of a sample were examined. To 30 ml of purified water, BIT (10 or 100 μg) was added. After 30 or 15 ml of organic solvent was added, the mixture was shaken for 5 min, and the partition behavior of BIT between the organic phase and the aqueous phase was observed. As organic solvents, ① cyclohexane, ② hexane, ③ diethyl ether, ④ four diethyl ether/hexane mixtures (4 : 1, 3 : 2, 2 : 3 and 1 : 4), ⑤ four diethyl ether/cyclohexane mixtures (4 : 1, 3 : 2, 2 : 3 and 1 : 4) were examined. The mixture containing equal volumes of water and diethyl ether/cyclohexane mixture (4 : 1 or 3 : 2) gave the highest transfer, at a rate of 74 to 78%, into the organic phase. Thus, it was decided to dissolve the adhesive in water and add an equal volume of diethyl ether/cyclohexane mixture (4 : 1), followed by repeating this procedure three times. For this procedure, the recovery rate was 90 to 98%. In addition, BIT was not extractable with cyclohexane or hexane.

When 2 ml of cyclohexane solution containing 10 or 100 μg of BIT was applied onto the silica gel cartridge column, and 30 ml of *n*-hexane was passed through, BIT was not eluted at all. Then, a total of 100 ml of acetone was passed in portions of 10 ml each, and 90% of BIT was eluted in the first two fractions (20 ml) but not in the subsequent fractions. Thus, it was decided to elute BIT from the column with 20 ml of acetone.

To 0.5 g of Sample No. 3 prepared with water, 20 or 200 μg of BIT was added, and the recovery test was conducted according to the procedure. The recovery rate was in a range of 85 to 90% (CV = 3.4%, $n = 3$).

Extraction and Cleanup for OIT: Solvents to extract OIT from an aqueous solution of a sample were examined. To 30 ml of purified water, OIT (11.9 or 119 μg) was added. After 30 ml of organic solvent was added, the mixture was shaken for 5 min, and the partition behavior of OIT between the organic phase and the aqueous phase was observed. As organic solvents, ① cyclohexane, ② hexane, ③ diethyl ether/cyclohexane mixture (1 : 1) and ④ diethyl ether/hexane mixture (1 : 1) were tested. For a mixture containing equal volumes of water and cyclohexane or n-hexane, the extraction rate into the organic phase was as high as 90 to 95%. Thus, it was decided to dissolve the adhesive in water and add an equal volume of cyclohexane. This was repeated three times. For this procedure, the recovery rate was 90 to 98%.

When 2 ml of cyclohexane solution containing 11.9 or 119 μg of OIT was applied onto a silica gel cartridge column and 30 ml of *n*-hexane were passed through, OIT was not eluted at all. Then, a total of 100 ml of hexane/acetone mixture (1 : 1) was passed in portions of 10 ml each, and 99% of OIT was eluted in the first two fractions (20 ml), but not in the subsequent fractions. Thus, it was decided to elute OIT with 20 ml of *n*-hexane/acetone mixture (1 : 1). In

addition, only 30% of OIT was extracted with *n*-hexane containing 20% acetone in the first two fractions.

To 0.5 g of Sample No. 3 prepared with water, 23.8 or 238 μg of OIT was added, and the recovery test was conducted according to the procedures. The recovery rate was 95 to 99% (CV = 2.9%, $n = 3$).

Extraction and Cleanup for TPN: Solvents to extract TPN from an aqueous solution of a sample were examined. To 30 ml of purified water, TPN (20 or 200 μg) was added. After adding 30 ml of organic solvent, the mixture was shaken for 5 min, and the partition behavior of TPN between the organic phase and the aqueous phase was observed. As organic solvents, ① cyclohexane and ② diethyl ether/cyclohexane mixtures (4 : 1 and 2 : 3) were examined. A high transfer rate of 95 to 97% was obtained for all solvents. Therefore, it was decided to extract the compound from an equivolume mixture of water and cyclohexane (1 : 1) because of the simplicity of the method.

When 2 ml of cyclohexane solution containing 2 or 20 μg of TPN was applied onto the silica gel cartridge column and 40 ml of n-hexane was passed through, no TPN was eluted. Then, 20 ml of *n*-hexane containing 1% acetone was passed through, and 99% of TPN was eluted. TPN was not eluted with an additional 20 ml of *n*-hexane containing 1% acetone. Thus, it was decided to elute TPN with 20 ml of *n*-hexane containing 1% acetone.

To 0.5 g of Sample No. 3 prepared with water, 200 μg of TPN was added, and the recovery test was conducted according to the procedures. The recovery rate was in a range of 98 to 103% (CV = 3.0%, $n = 3$).

Analytical Results

Table 2 shows the analytical results of the adhesives by the procedures mentioned above. These antimicrobial agents were detected in most of the products. OIT and TPN were detected at a high concentration in Sample No. 7, especially, TPN was detected at the order of mg/g. Analysis of the antiseptics attached to Sample No. 3 revealed that the antiseptics were composed of MIT and Cl-MIT.

Figure 2(A) shows FPD-GC for the standard solutions of four isothiazolone antimicrobial agents, while Fig. 2(B) shows an FPD-GC for Sample No. 1 (a mixture of test solutions prepared for the quantitative analysis of each compound). Figure 3 shows an ECD-GC of Sample No. 7. Figure 4 shows the mass spectra of each antimicrobial agent in the samples.

Table 2. Analytical Results of Antimicrobial Agents in Non-Formalin Adhesives for Wallpaper (μg/g)

Sample No	MIT	Cl-MIT	BIT	OIT	TPN
No. 1	10.4	26.5	N.D.	11.0	N.D.
No. 2	4.4	13.7	N.D.	5.9	N.D.
No. 3	N.D.	N.D.	N.D.	N.D.	N.D.
No. 4	8.1	20.3	N.D.	N.D.	N.D.
No. 5	8.9	14.6	N.D.	N.D.	N.D.
No. 6	7.6	17.7	N.D.	N.D.	N.D.
No. 7	5.9	18.2	N.D.	133.0	**2480**

N.D.: Not detected. The unit of the measurement concentration is μg/g.

Although MIT and Cl-MIT have been used as antiseptics for cosmetics for a long time and the negative data on contact allergenicity have been published,[17] many cases of skin impairments due to these compounds have been reported.[18–23] While there is a report on the analysis of methylisothiazolone formulation (Kethon C.G., *etc*.) by HPLC,[5] analytical methods for commercial products such as cosmetics containing these chemicals have hardly been reported. Therefore, the actual states of their utilization and concentrations are not known. The present study clarified that these chemicals are actually used as antiseptics for wallpaper adhesives.

An antimicrobial agent, BIT, is used as an antiseptic or a fungicide in paints, leathers, cutting oils and printing ink.[24] This agent is known to have skin irritability and skin-sensitization activity.[25–27] Numerous cases among paint manufacturers[28] and the employees in the printing industry, wall repapering, metal processing and chemical industries[29–35] have reported allergic symptoms, probably due to BIT. In the study of subacute toxicity test in rats, a weight decrease in the liver and pituitary gland,[36] thickening of the anterior stomach and thymus atrophy[37] have been reported. However, there are few reports of analytical methods for this chemical.

OIT is a compound widely used as an antiseptic in paints, timbers and plastics,[38] and we have detected it qualitatively in commercially available household paints.[39] Although no data on contact allergenicity of OIT has been reported,[38] there are some case reports on skin damage.[40–43] But there are no reports on analytical methods, and it was difficult to obtain a standard product of OIT. As mentioned above, the concentration of OIT in the antiseptics sold for industrial use was used as the standard reference.

One of the organic chlorine antiseptics, TPN, has

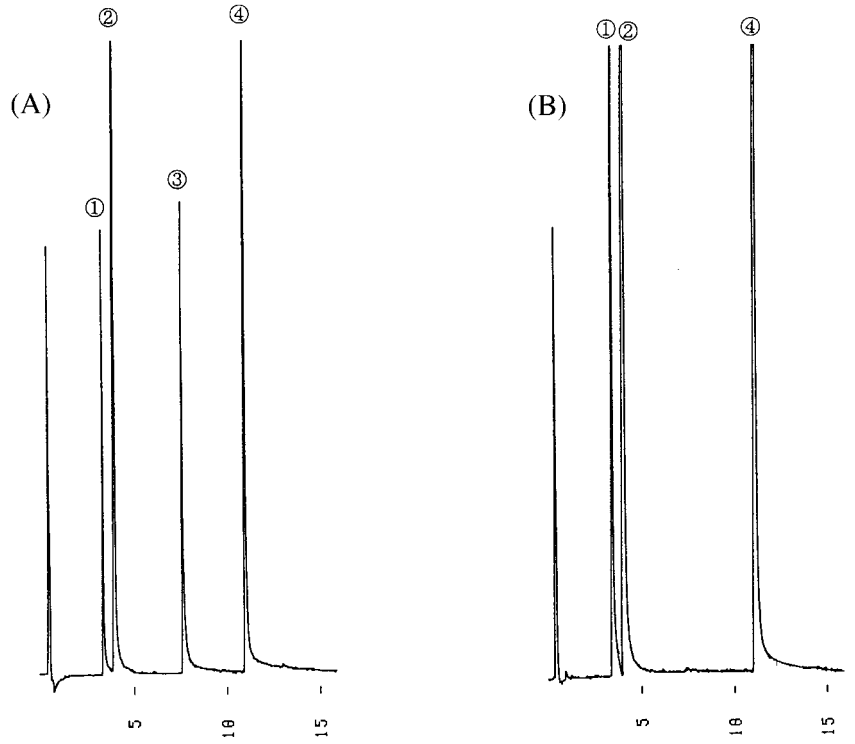

Fig. 2. FPD Gas Chromatograms of a Standard Solution of Isothiazolones (A) and Sample No. 1 (B)

①MIT: 2-methyl-4-isothiazolin-3-one, ②Cl-MIT: 5-chloro-2-methyl-4-isothiazolin-3-one, ③BIT: 1,2-benzisothiazolin-3-one, ④OIT: 2-*n*-octyl-4-isothiazolin-3-one, Sample No. 1 (a mixture of the testing solutions prepared for the quantitative analysis of each compound). Operating conditions of FPD-GC are given in Table 1.

been widely used as an antiseptic or fungicide for agriculture, paints and timber under the market names of Chlorothalonil or Daconil.[44,45] The lethal dose of TPN by oral administration in rats was 10 g/kg, and the chemical was toxic to fishes and irritable to human eyes and skin.[45] The International Agency for Research on Cancer (IARC) has classified TPN as Group 3 (materials of which carcinogenicity have not been reported in man), and the Environment Agency has defined it as a substance subject to the Pollutant Release and Transfer Register (PRTR). The occurrence of allergic symptoms in woodworkers,[46] painters[47] and gardeners[48] has been reported. In a recent study of contact allergenicity in a guinea pig maximization test (GPMT),[49] TPN was reported to be an extremely strong contact sensitizer.[50]

There are many reports on analytical methods for TPN in agricultural products,[5–9] soils,[10,11] water[12,13] and air.[14–16] TPN is classified as a semivolatile organic compound by WHO criteria,[51] and its detection in air has been reported.[14–16] Therefore, there is a possibility that workers in wall repapering might be exposed to a high concentration of TPN, not only through the skin but also the respiratory tract if the compound is used at a high concentration, as seen for Sample No. 7. Moreover, residents may be exposed to TPN in the air since wallpapers are used in large areas. Allergic contact dermatitis is known to be inducible even by exposure to a low concentration, once the skin is sensitized by a high concentration of the compound. Multiple chemical sensitivities are also reported to occur by slight re-expo-

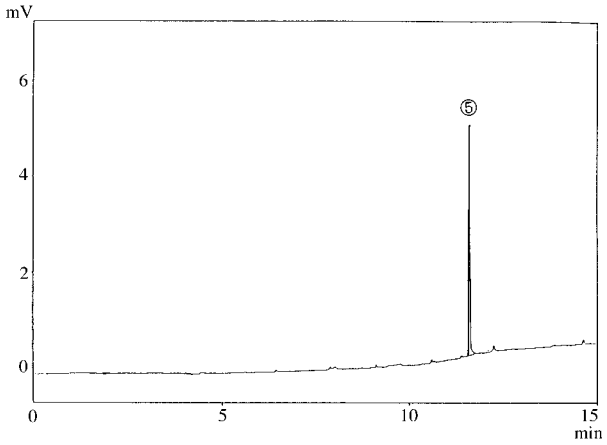

Fig. 3. ECD Gas Chromatogram of Sample No. 7

⑤TPN: 2,4,5,6-tetrachloroisophthalonitrile. Operating conditions of ECD-GC are given in Table 1.

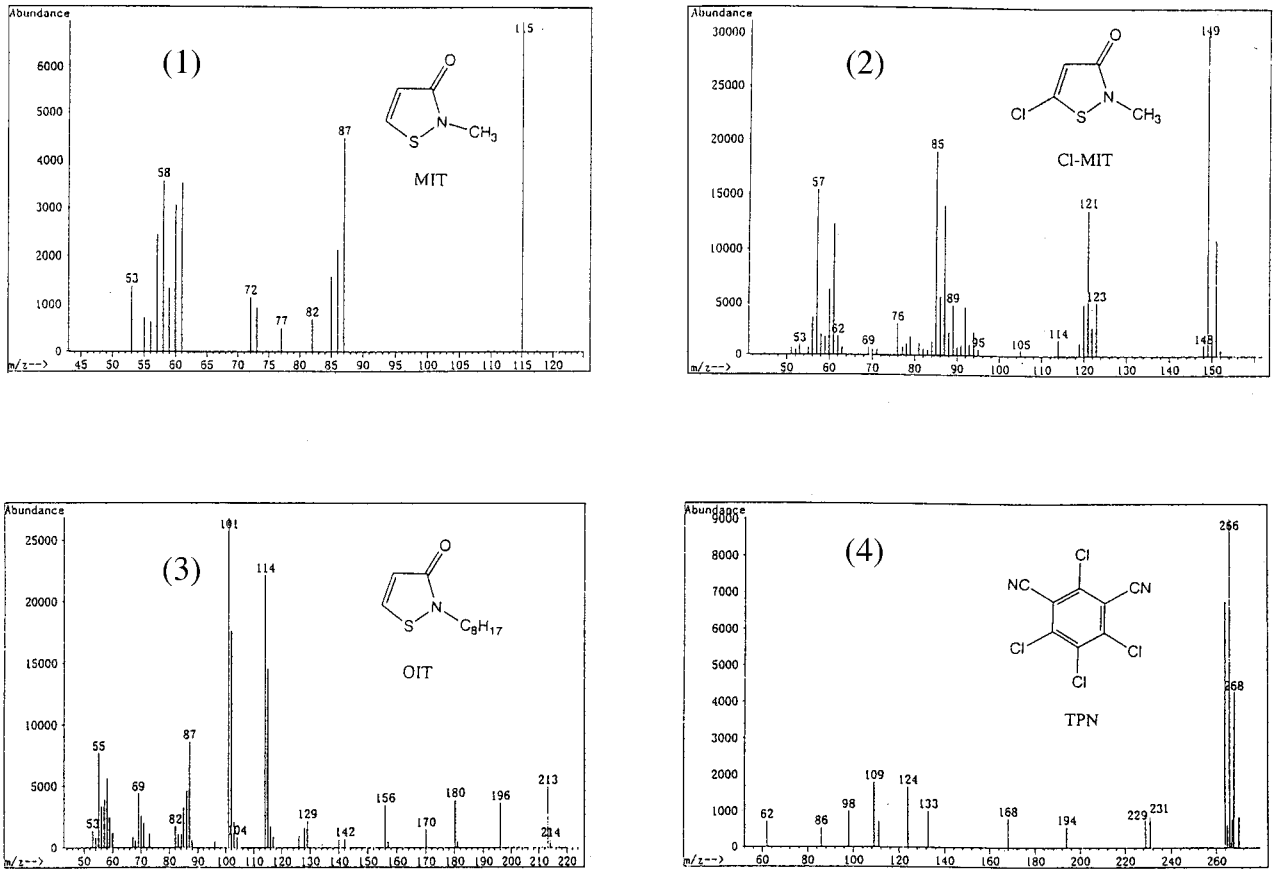

Fig. 4. Mass Spectra of Each Confirmed Antimicrobial Agent in the Samples by GC/MS.

(1) Mass spectrum of MIT detected from Sample No. 1. (2) Mass spectrum of Cl-MIT detected from Sample No. 1. (3) Mass spectrum of OIT detected from Sample No. 1. (4) Mass spectrum of TPN detected from Sample No.7. Operating conditions of GC/MS are given in Table 1.

sure after previous exposure at a high concentration.[1–4] Although there is no specification for the chemicals in the package insert of Sample No. 7, except description that chemicals of safety and low toxicity should be used, it is not likely that the safety of TPN is higher than that of formalin. Therefore, it is recommended to avoid the use of TPN at such a high concentration as seen in the case of Sample No. 7.

These results suggest that, in the use of non-formalin adhesives as building materials with safety and ecological properties, isothiazolone antimicrobial agents and TPN were mixed as substituting chemicals. It is not likely that these are more safe than formalin in some conditions of exposure. Thus, it is necessary to specify the kind and the concentration of drug used. Furthermore, for infants, the elderly and hypersensitive patients who are more susceptible to agents, the application must be made at each appropriate concentration in consideration of exposure conditions (area of contact, exposure time and distance to contact, *etc*.). In the future, it will be necessary to evaluate the toxicities of these drugs as well as their exposure effects.

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