PEER-REVIEWED

High-Performance Liquid Chromatography Identification, Quantification, and Fractionation of a Suspect Allergen, 4-Chloro-3-methylphenol, in an LLNA-Positive Metalworking Fluid

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Editor's Note: Biocides are added to metalworking fluids to prolong the life of the fluid, but there is a potential side effect of causing allergic reactions in users. This month's Editor's Choice paper investigates methodology to separate, identify and quantify one suspected allergen. By creating separate fractions and reconstituting the samples, reactions to suspected allergens are relatively straightforward. The value in performing such analysis, to ensure performance-enhancing chemistry does not have a detrimental health affect, is significant to the formulators and end-users of these products.

Evan Zabawski, CLS
Editor

KEY WORDS

Metalworking Fluids; Maintenance; Safety: Toxicology; Biocides; Hygiene; Lubricant Microbial Decomposition; Chemical Contamination; Cleanliness; Liquid Chromatography; Gas Chromatography; Spectroscopy

ABSTRACT

A metalworking fluid (MWF) was obtained that produced an allergic reaction in the local lymph node assay (LLNA) with an EC3 = 4%, the EC3 being the estimated concentration needed to provoke a 3-fold allergy response. High-performance liquid chromatography (HPLC) was used to separate, identify, and isolate the suspected allergen. The biocide, 4-chloro-3-methylphenol, was detected in the MWF as a chromatographic peak matching the retention time of an external standard. The technique of standard addition was used to quantify and confirm the presence of 4-chloro-3-methylphenol at about 1% (w/w). Preparative HPLC was used to fractionate 1 gram of MWF separating the 4-chloro-3-methylphenol fraction from the remaining MWF. The two mobile-phase solutions were concentrated back into an MWF and a 4-chloro-3-methylphenol fraction. The original MWF and the reconstituted 4-chloro-3-methylphenol and MWF fractions were also analyzed by gas chromatography–mass spectrometry to confirm the isolation of the biocide.

INTRODUCTION

Estimates indicate that more than 13 million workers in the United States are potentially exposed to chemicals that can be absorbed through the skin. A worker's skin may be exposed to hazardous chemicals through direct contact with contaminated surfaces, deposition of aerosols, immersion, or splashes. When substantial amounts of chemicals are absorbed, systemic toxicity can result. Contact dermatitis can also result when chemicals are absorbed through the worker's skin and is one of the most common chemically induced occupational illnesses, accounting for 10–15% of all occupational illnesses at an estimated annual cost of at least $1 billion (NIOSH (1)).

Some 1.2 million workers in machine finishing, machine tooling, and other metalworking and metal-forming operations are potentially exposed. Workers can be exposed to the fluids by breathing aerosols generated in the machining process, or through skin contact when they handle parts, tools, and equipment covered with the fluids. The National Institute for Occupational Safety and Health (NIOSH) defines MWF aerosol as the mist and all contaminants in the mist generated during grinding and machining operations involving products from metal and metal substitutes (NIOSH (1)).

Worldwide usage of MWFis estimated to be 2 billion liters per year (Cheng, et al. (2)). MWFs are divided into four classes based on their oil and water content: insoluble, soluble, semi-synthetic, and synthetic. Insoluble (or straight) oil MWFs function mostly as lubricants and are not diluted with water. Similar to straight oil MWFs, soluble oil MWFs are used to cool and lubricate tool surfaces but are diluted with water. Semi-synthetic MWFs contain small amounts of oil and additives, while synthetic MWFs have no oil at all in its formula (NIOSH (3)).

Besides water and oil, MWF scan contain hundreds of different compounds and additives such as amines, surfactants, emulsifiers, detergents, and biocides. These intentional additives are chemicals used to modify the formula improving or enhancing its performance (Sheehan (4)). Biocides are oftentimes used to destroy the variety of microorganisms or fungi that can survive in MWFs (Cheng, et al. (2)). If gram-negative bacteria are present, endotoxins are released. These endotoxins can contaminate MWFs and may enhance the allergic responses in exposed workers (Lim, et al. (5)).

MWF exposure assessment has been a priority research area at the National Institute for Occupational Safety and Health (NIOSH) as evident by research dating back to the early 1970s (Glaser, et al. (6)). In a 1998 document, Criteria for a Recommended Standard: Occupational Exposure to Metalworking Fluids, NIOSH recommended that the level of exposure for any given worker should not exceed 0.4 mg/m^3 of air (thoracic particulate mass) as a time-weighted average concentration for up to a 10-h day during a 40-h week. Gravimetric and infrared spectrophotometric techniques, published in the NIOSH Manual of Analytical Methods as Methods 0500, 5024, and 5526, can be used to estimate MWF exposure (NIOSH (7)). When using gravimetric or infrared techniques, the results are limited to estimating the mass or concentration level of the MWF without the identification of its chemical composition (Verma, et al. (8); Raynor, et al. (9)). Because chemicals have very different toxicities, the toxicity of an exposure cannot be assessed without knowledge of the chemical composition.

Little is known about the exact chemical makeup of each MWF because of the competitiveness of the industry and trade secrets. A method that can separate and identify the components of a MWF is needed to assist workers who develop allergic contact dermatitis. Analytical chromatography is a technique used to separate complex mixtures. In HPLC, mixtures are separated on a chromatography column and elute off as purified components. HPLC has been utilized to identify the presence of contact allergens and to isolate allergen bands or fractions (Lee, et al. (10); Wahl, et al. (11)).

In this project, semi-preparative HPLC was used to identify and isolate 4-chloro-3-methylphenol from a metalworking fluid. About 1000 mg of the sample is needed for a local lymph node assay (LLNA). 4-Chloro-3-methylphenol has also been used in skin cosmetics. Andersen, et al. (12) determined that 4-chloro- 3-methylphenol was a sensitizer in guinea pigs using 5 topical preparations and the cumulative contact enhancement test. However, that test may have overestimated the sensitizing potential of 4-chloro-3-methylphenol because later studies using human skin patch testing showed only 2% of the 1462 subjects were allergic to this agent. They concluded that the results from guinea pig allergy tests cannot stand alone but have to be validated by other sources of information. Later in 1997, an expert panel reported in the International Journal of Toxicology a "Final Report on the Safety Assessment of p-Chlorom-Cresol" that concluded the available data was insufficient to support the safety of 4-chloro-3-methylphenol for use in cosmetic products (Final Report (13)). As with cosmetics, the safety assessment of 4-chloro-3-methylphenol in metalworking fluids that contact the skin has insufficient data, and thus this study is to provide more data for safety assessment in metalworking fluids.

EXPERIMENTAL

Instrumentation

The HPLC system consisted of HP-1050 modular units that included an injector, a UV detector, and a quaternary solvent pump (Agilent Technologies, Palo Alto, CA). UV light adsorption was monitored at 254 nm for analyte detection. The column was a 10 mm × 300 mm XTerra ® Prep MS-C18 (Waters, Milford, MA). The column had a 10 μm particle size, a 0.65 cm^3/g pore volume, and an average pore diameter of 113 Å. Mobile phase flow rate was 5.0 mL/min at room temperature, and the injection volume was 100 μL. This method used a ternary mobile phase system, three solutions. The Dionex AI-450 Chromatography Data Acquisition Software was run on a Microsoft Windows PC system (Dionex Corp., Sunnyvale, CA).

Chemicals

The mobile-phase solvents were ACS-HPLC grade hexane, 2-propanol, and acetonitrile from Burdick & Jackson (Honeywell Corp., Morristown, NJ). Mobile phase A used purified water from a Milli QTM Nanopure Water System Model D4751 (Barnstead Int., Dubuque, Iowa). Formic acid, p.a. (pro analysis) grade, was added to mobile phase A at 0.1%, v/v, (Acros Chemicals, NJ). Mobile phase B was 33.3% 2-propanol and 66.6% acetonitrile. Mobile phase C was 33.3% 2-propanol and 66.6% hexane. A "universal solvent mixture" (USM) solution of methanol, 2-propanol, and hexane (1:1:1 v/v/v) was prepared to dissolve the MWF concentrate.

HPLC Identification and Quantification of 4-Chloro-3-Methylphenol in MWF

A MWF sample was obtained from the National Toxicology Program, NTP. A standard of 4-chloro-3-methylphenol (chlorocresol) was obtained from Chem Service, Inc. (West Chester, PA). The presence of 4-chloro-3-methylphenol in the MWF was determined by matching chromatographic peak retention times of the samples against the external standards. Then a standard addition technique was performed to help confirm the identity of the peak and estimate the original amount of 4-chloro-3-methylphenol in the MWF. Four solutions were prepared for standard addition analysis: A, B, C, and D. Solution A consisted of only 4-chloro-3- methylphenol external standard, 7.80 mg diluted in 10.00 mL of USM (0.780 mg/mL). Solution B consisted of only MWF, 1.878 g diluted in 25.00 mL of USM (75.12 mg/mL). This MWF solution was sonicated at 55°C and its density was determined. In solution C, 1.23 mg of 4-chloro-3-methylphenol standard was added to 1.50 mL of MWF solution (+0.82 mg/mL). Solution D consisted of 5.3 mg of 4-chloro-3-methylphenol added to 1.50 mL of MWF solution (+3.53 mg/mL). Using HPLC analysis, the retention times and peak areas of the 4-chloro-3-methylphenol were determined for each solution. Injection volume was 100 μL (0.1 mL). Table 1 lists the mobile-phase mixing program used for the ternary mobile phase gradient program.

Table 1 | **Mobile-Phase Gradient Program of the High-Performance Liquid Chromatography Method for Analysis, Fingerprinting, and Quantification of Metalworking Fluid Components.**

Time (Min)	Mobile Phase % A	Mobile Phase % B	Mobile Phase % C	Flow (mL/min)
0	100	0	0	0.1
0.1	100	0	0	5.0
10.0	50	50	0	5.0
60.0	0	100	0	5.0
90.0	0	0	100	5.0
90.1	0	100	0	5.0
100	0	100	0	5.0
100.1	100	0	0	5.0
110	100	0	0	5.0

HPLC Fractionation of a Metalworking Fluid

To fractionate the 4-chloro-3-methylphenol from the MWF, the HPLC method was used, but with a truncated mobile phase program to save time as found in Table 2. The injector was programmed for multiple 100-μL injections of MWF solution, 10 times for a total of 1.0 mL of solution, onto the column in order to deliver 75 mg of MWF. This was done by programming 10 injections per vial with a run stop time of 0.1 min for the first 9 injections and then manually changing the run stop time to 60 min after the ninth injection. The

Table 2 | **Mobile-Phase Gradient Program for the Semi-Preparative High-Performance Liquid Chromatography (HPLC) Fractionation Method.**

Time (Min)	Mobile Phase % A	Mobile Phase % B	Mobile Phase % C	Flow (mL/min)
0	100	0	0	0.1
0.1	100	0	0	5.0
10.0	50	50	0	5.0
20.0	40	60	0	5.0
20.1	0	100	0	5.0
30.0	0	100	0	5.0
30.1	0	0	100	5.0
40.0	0	0	100	5.0
40.1	0	100	0	5.0
50.0	0	100	0	5.0
50.1	100	0	0	5.0
60.0	100	0	0	0.1

HPLC effluent containing 4-chloro-3-methylphenol was diverted and captured during a predetermined retention time window into a 10-mL test tube. It took 17 HPLC runs to process 1,275 mg of MWF into two fractions, a 4-chloro-3-methylphenol fraction and an MWF fraction without 4-chloro-3-methylphenol. To determine the efficiency of this method, cool-oncolumn gas chromatography–mass spectrometry (COC-GC-MS) analysis was done on each fraction for 4-chloro-3-methylphenol.

RESULTS

Quantification of Biocide by Standard Addition Technique

HPLC analysis of solution A, the standard of 4-chloro-3-methlyphenol, produced chromatogram A in Figure 1 with a peak at 26.62 min. The 0.078 mg of 4-chloro-3-methlyphenol that produced the peak in chromatogram A was from an injection volume of 100 µL with a concentration of 0.780 mg/mL. Solution B, containing only MWF, produced a chromatogram with multiple peaks, but one was at 26.60 min. The MWF sample solution contained 0.0751 g/mL, resulting in 7.51 mg of MWF being injected onto the column in chromatogram B. Solution C with standard addition resulted in chromatogram C with an increased height of the peak at 26.62 min. The increased peak height in solution C was the result of an added 0.082 mg or 0.82 mg/mL of 4-chloro-3-methylphenol. Finally, solution D with even more standard added resulted in an even higher peak area response at 26.62 (*see Figure 1*).

The UV absorption peak area data for 4-chloro-3- methyphenol from chromatograms a, b, and c were analyzed by least squares linear regression for their average relationship to the amount of 4-chloro-3-methylphenol in the MWF and the data was plotted as shown in Figure 2. The linear equation found was Y = 99.7E6X+8.14E6. The X intercept at Y=0 estimated the amount of 4-chloro-3-methlyphenol in the MWF at 0.082 mg. There was 7.512 mg of MWF injected. Therefore, there was 1.1% 4-chloro- 3-methlyphenol (w/w) in the MWF.

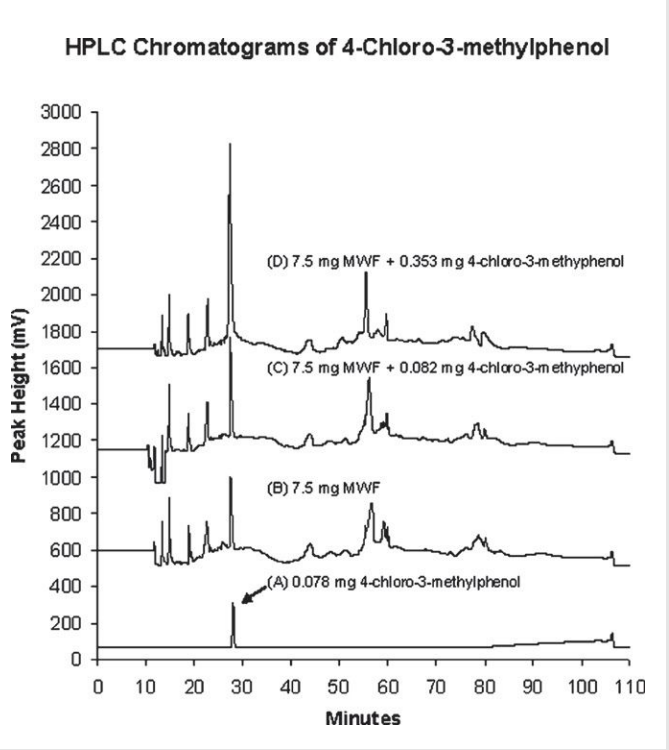

Figure 1 | **Overlay of four HPLC chromatograms used to quantify 4-chloro-3-methylphenol in MWF. A) Chromatogram was from a 0.078 mg 4-chloro-3-methylphenol standard. B) Chromatogram was from 7.5 mg of MWF. C) Chromatogram was from 7.5 mg of MWF with 0.082 mg of 4-chloro-3-methylphenol added. D) Chromatogram was from 7.5 mg of MWF with 0.350 mg of 4-chloro-3-methylphenol added. The retention time for 4-chloro-3-methylphenol was identified at 26.62 min from standard.**

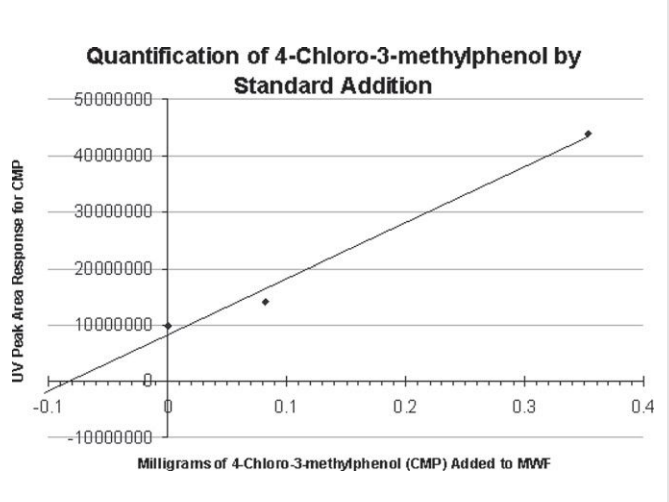

Figure 2 | **Line graph shows the quantification of 4-chloro-3-methylphenol by the standard addition technique. Least squares linear regression analysis of the 3 response data points against the amount of 4-chloro-3-methyphenol added resulted in a linear equation for peak area as a function of amount of analyte. The point where this line intercepts the X axis determined the amount of biocide in the MWF at 0.082 mg of 4-chloro-3-methylphenol in 7.512 mg of MWF or 1.1%.**

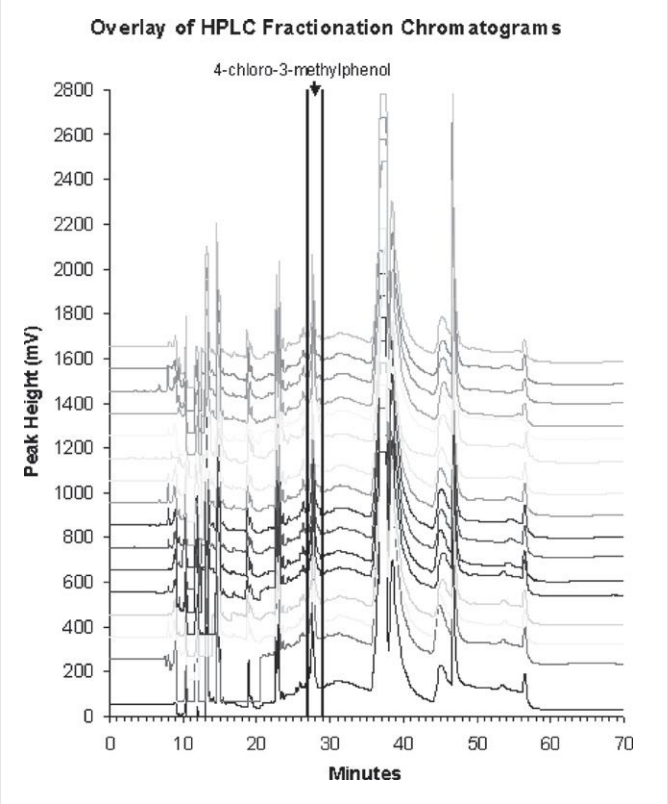

Figure 3 | **This is an overlay of the 17 runs. The runs remain in order with the first one being the bottom chromatogram. With exception to the first chromatogram, the peak heights for each run were offset by 100 units over the previous run. The two vertical lines represent the fractionation window of approximately 27.0 to 29.0 min.**

HPLC FRACTIONATION OF METALWORKING FLUID

Figure 3 shows an overlay of the 17 chromatograms produced during the fractionation process. The two vertical lines that overlay the chromatograms intersecting the X axis at 27.0 and 29.0 represent the collection window for the 4-chloro-3-methylphenol peak. The mobile phase eluent containing 4-chloro-3-methylphenol was collected from each of the 17 runs between approximately 27.0 and 29.0 min and pooled. The mobile phase before and after the window was also collected and pooled. The two pooled fractions were concentrated using a nitrogen evaporator at 60°C to a final volume of 1275 μL back to the MWF samples' original density of 1.0 g/mL.

Cool-on-Column Gas Chromatography Mass Spectrometry Analysis of Metalworking Fluid

The original MWF solution, the isolated 4-chloro-3-methylphenol fraction, and the MWF fraction minus the 4-chloro-3-methylphenol fraction were analyzed by COC-GC-MS. The total ion current (TIC) chromatogram resulting from COC-GCMS analysis of the 4-chloro-3-methylphenol fraction showed only one peak after the solvent peak, Figure 4.

The identity of 4-chloro-3-methylphenol in that peak was confirmed by mass spectrometry through the high correlation of its mass spectrum with that of the library spectrum (*see Figure 5*). The identified parent ion at m/z of 142 was used

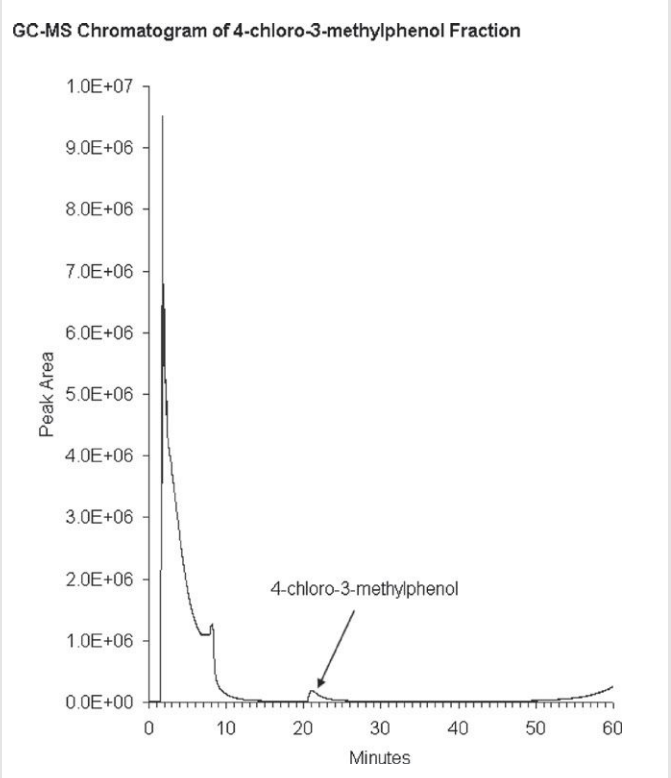

Figure 4 | **The suspect allergen was found in GC-MS chromatogram, the peak located at approximately 21 min.**

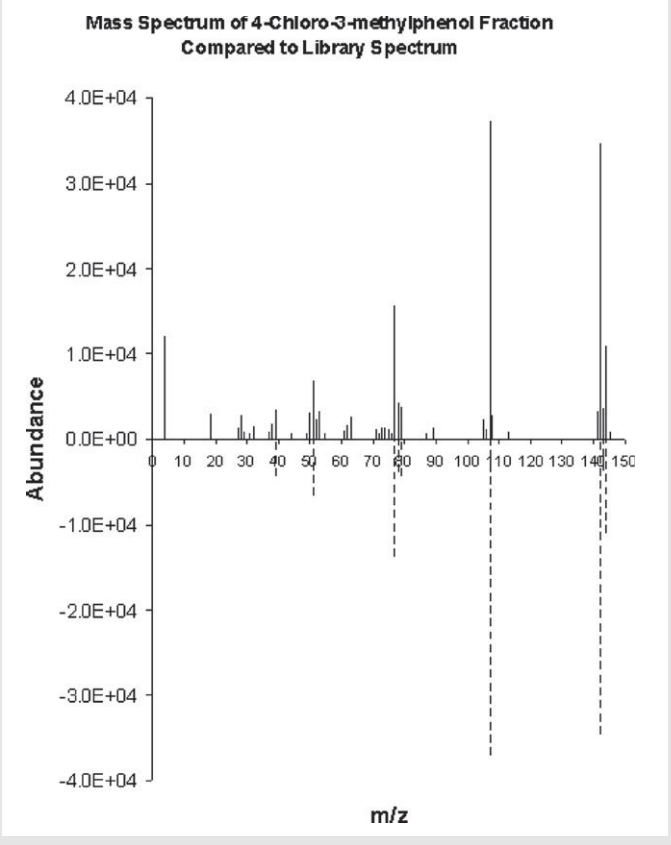

Figure 5 | **The mass spectrum of the 4-chloro-3-methylphenol peak at 21 minutes (top) is shown. The reference mass spectrum of 4-chloro-3-methylphenol (bottom) is from the National Institute of Advanced Industrial Science and Technology (AIST).**

in the future for more specific selected ion monitoring (SIM) analysis.

In Figure 6, the top overlay of the three TIC chromatograms compared the 4-chloro-3-methylphenol content in each fraction. TIC chromatograms result from the responses of rapidly repeated mass spectrum scans from 2 to 800 amu during the chromatogram. The chromatogram from the 4-chloro-3-methylphenol fraction showed only one peak at the expected retention time of this analyte. The chromatograms of the original MWF sample and MWF without 4- chloro-3-methylphenol showed parallel matrix profiles; however, TIC detection also responded to the matrix coeluting at the retention time of 4-chloro-3-methylphenol and negated proving that the biocide was totally removed. Incidentally, a peak at about 18 min in the original MWF suffered loss during reconstitution. This peak was a pine scent component, terpineol, but otherwise the MWF fraction minus the 4-chloro-3-methylphenol appeared to contain all the other detectable components in the original matrix. The lower overlays in Figure 6 of the more specific SIM chromatograms extracted from the TIC chromatogram, showed that only the original MWF and the 4-chloro-3-methylphenol fraction contained the biocide and that there was no trace of 4-chloro-3-methylphenol in the "MWF fraction minus the 4-chloro-3-methylphenol"; i.e., it was completely resolved from the original MWF.

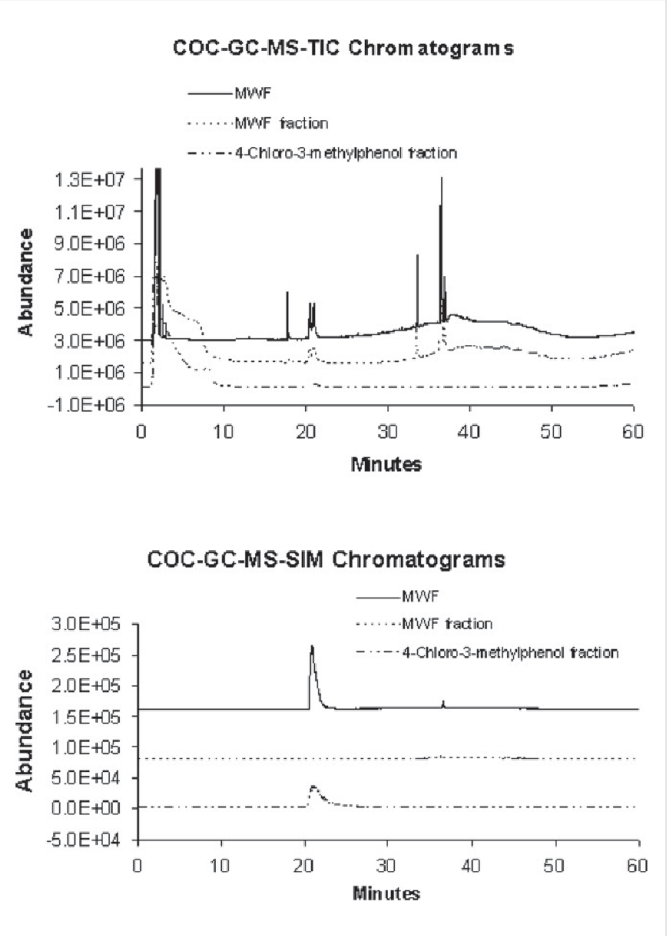

Figure 6 | **The chromatograms of the original MWF sample, the MWF fraction, and the 4-chloro-3-methylphenol (chlorocresol) fraction are shown. The upper chromatograms are of the sample using the cool-on-column injection gas chromatography with conventional electron impact mass spectrometry detection monitoring total ion current (COC-GC-MS-TIC). The lower chromatograms are the same chromatograms but with selective ion monitoring for the chlorocresol 142 m/z ion (COC-GC-MS-SIM).**

CONCLUSION

An HPLC-UV method was developed that could separate, measure, and isolate the biocide, 4-chloro-3-methylphenol, in the MWF. The standard addition technique confirmed and quantified the content of 4-chloro-3-methylphenol in the MWF, and identified its retention time. COC-GC-MS analysis confirmed the presence of 4-chloro-3-methylphenol in the MWF, its absence in the MWF fraction, and its presence in the 4-chloro-3-methylphenol fraction. Multiple injections maximized the loading capacity of the semi-preparative column, 75mg. It took 17 HPLC runs to fractionate 1275 mg of MWF for LLNA into two separated fractions. The semi-preparative HPLC method resulted in reproducible chromatograms for the seventeen fractionations of MWF. One HPLC run was sufficient to fractionate a metalworking fluid for the COC-GC-MS analysis of the component. Seventeen HPLC runs were needed to fractionate the metalworking fluid for LLNA that requires

1000 mg of sample. For LLNA, the two recovered fractions, the 4-chloro-3-methylphenol and the remaining MWF, were reconstituted by nitrogen evaporation to a volume of 1275 μL; i.e., to its original concentration. HPLC can fractionate contaminated MWFs for COC-GC-MS analysis of trace impurities that requires enrichment.

DISCUSSION

Previous studies have shown that this MWF was a potential cause of contact dermatitis and analysis of the fluid showed it to contain 4-chloro-3-methylphenol, a suspected allergen. Using HPLC, the 4-chloro-3-methylphenol was separated, identified, confirmed, quantified, and isolated from the MWF. This current analytical method differs from IH methods where gravimetric analysis was used to determine the total mass concentrations of the MWF in air for assessment of worker exposure. In order to confirm that 4-chloro-3-methylphenol is an allergen, the LLNA needs to be performed on the separated fractions. If the fraction containing the suspect allergen produces the same allergenic response and the reconstituted MWF does not, then the allergen was identified. The HPLC method using Table 1 mobile phase conditions and with a smaller analytical column has been routinely used in the laboratory also for lower volume injections with similar success.

SUGGESTIONS

To decrease the time it takes to process the desired amount of sample for LLNA, a larger HPLC preparative column and system can be used. The advantage of this system is that it used a standard HPLC analytical pump system, but the disadvantage was that the analytical pump systems are not designed for large preparative column flows and so the semi-preparative column was a compromise that required multiple runs. There are preparative HPLC systems commercially available that could have processed a gram in a single chromatogram. To decrease evaporation time, more than one evaporator can be utilized. Furthermore, this HPLC method appears to be versatile and applicable to many complex mixtures, and the methodology may be used to identify and remove allergens from other complex mixtures. In the medical field specifically, HPLC in combination with the LLNA approach could be used to help identify and remove allergens that cause allergic contact dermatitis (ACD) in individuals.

DISCLAIMER

Mention of company names and/or products does not constitute endorsement by the CDC. The findings and conclusions of this report are those of the author(s) and do not necessarily represent the views of the National Institute for Occupational Safety and Health. TLT

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