Determination of *p*-Phenylenediamine and Related Antioxidants in Rubber Boots by High Performance Liquid Chromatography. Development of an Analytical Method for *N*-(1-Methylheptyl)-*N*′-phenyl-*p*-phenylenediamine

Yoshiaki Ikarashi* and Masa-aki Kaniwa

Division of Medical Devices, National Institute of Health Sciences, 1–18–1, Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan

(Received June 16, 2000; Accepted August 4, 2000)

Although *p*-phenylenediamine (PPD) and related compounds have been used as antioxidants in rubber products, they commonly display sensitizing properties and have been associated with contact dermatitis. *N*-Substitution of PPD influences the sensitization potential, so it is important to examine which PPD antioxidants are used in commercially available rubber products, to prevent the occurrence of contact dermatitis in sensitized patients. In this study we developed a method for the determination of PPD derivatives, such as *N*-(1-methylheptyl)-*N*′-phenyl-*p*-phenylenediamine (MHPPD), *N*-isopropyl-*N*′-phenyl-*p*-phenylenediamine (IPPD) and *N*-1,3-dimethylbutyl-*N*′-phenyl-*p*-phenylenediamine (DMBPPD), using high performance liquid chromatography (HPLC), and investigated the PPD antioxidant content of rubber boots used by farmers. The PPD derivatives were extracted from rubber boots with acetone : chloroform (1 : 1). The extract was loaded then on to a silica–gel column, and eluted with 50 ml of diethylether : hexane mixtures (5 : 95, 10 : 90, 20 : 80 and 50 : 50) in that order. MHPPD and DMBPPD were eluted in the diethylether : hexane (10 : 90) fraction. The recovery of MHPPD by fractionation was 95 ± 8% (n = 5). IPPD was detected in the diethylether : hexane (20 : 80) fraction. Each fraction was evaporated, and the residue was dissolved in dichloromethane and subjected to HPLC with an ODS column and a UV detector (detection wavelength 290 nm). The mobile phase was methanol : water (85 : 15). After fractionation, the retention times of these PPD derivatives were found not to overlap with other rubber additives. The linear calibration curves for MHPPD, DMBPPD and IPPD were obtained over the range of 0.1–400 μg/ml. Using this method, eight types of rubber boot used by farmers were analyzed. MHPPD was not found in any of the rubber boots, but DMBPPD and IPPD were detected.

Key words —— *N*-(1-methylheptyl)-*N*′-phenyl-*p*-phenylenediamine, antioxidant, rubber, HPLC, *p*-phenylenediamine, boots

INTRODUCTION

p-Phenylenediamine (PPD) and related compounds have been used as antioxidants in rubber products.[1] Because of their color, the use of these PPD derivatives has been limited to black rubber products, such as boots or tires.[1] Accordingly, people engaged in farming frequently come into contact with these chemicals by wearing rubber boots.

PPD derivatives commonly display sensitizing properties and are associated with contact dermatitis.[2] PPD shows strong sensitization potency and has been used as a positive standard sensitizing chemical in the guinea pig maximization test (GPMT).[3] *N*-Substitution of PPD increases or decreases the sensitization potential, and the length of the chain of the alkyl substituent often has an effect on the sensitization potential.[4,5] *N*-Isopropyl-*N*′-phenyl-*p*-phenylenediamine (IPPD), which has been detected in farmers' boots, has been associated with the development of allergic contact dermatitis,[6–8] while *N*-1,3-dimethylbutyl-*N*′-phenyl-*p*-phenylenediamine (DMBPPD) also has a high sensitization potential as well as IPPD in the GPMT.[4,9]

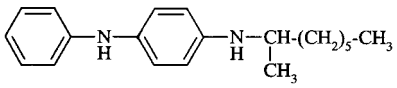
N-(1-Methylheptyl)-*N*′-phenyl-*p*-phenylenediamine (MHPPD) is also an antioxidant for black rubber products.[10,11] Shimizu *et al*. described the weaker sensitization potential of MHPPD in the

*To whom correspondence should be addressed: Division of Medical Devices, National Institute of Health Sciences, 1–18–1 Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan. Tel.: +81-3-3700-1141; Fax: +81-3-3707-6950; E-mail: ikarashi@nihs.go.jp

GPMT, compared with IPPD and DMBPPD,[12] and so MHPPD has been used as an alternative to IPPD or DMBPPD. It is important to know the type and/or quantity of PPD derivatives that have been used in commercially available rubber products. However, there is no published report of any analytical method or data on MHPPD in rubber products. The purpose of this study is to develop an analytical method for PPD derivatives, such as MHPPD, in rubber products.

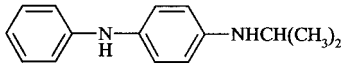
In previous studies, the determination of PPD antioxidants (IPPD and DMBPPD) in rubber products was carried out by gas chromatography (GC).[6,13,14] However, many peaks were found in the GC chromatograms using a flame ionization detector, GC/mass spectrometry (GC/MS) was needed for the qualitative analysis of each PPD derivative. Furthermore, the rubber additives were generally highly reactive, and some decomposed on heating. High performance liquid chromatography (HPLC) has been adopted as a popular method for the determination of phenolic antioxidants in various polymers[15,16] and, during HPLC, test chemicals are not heated above room temperature. Each PPD derivative has a characteristic ultraviolet absorption spectrum, and qualitative analysis is possible by selection of a suitable detection wavelength. Therefore, we decided to use HPLC for the determination of PPD derivatives. In addition, we achieved a chromatographic separation of PPD derivatives and obtained analytical data on PPD derivatives in rubber boots used by farmers.

N-(1-Methylheptyl)-*N*′-phenyl-*p*-phenylenediamine (MHPPD)

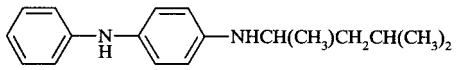


p-Phenylenediamine (PPD)

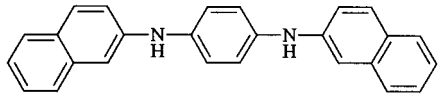
N-Isopropyl-*N*′-phenyl-*p*-phenylenediamine (IPPD)



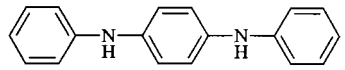
N-1,3-Dimethylbutyl-*N*′-phenyl-*p*-phenylenediamine (DMBPPD)



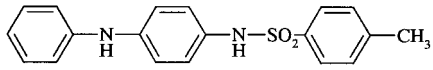
N,N′-Di-2-naphtyl-*p*-phenylenediamine (DNPD)



N,N′-Diphenyl-*p*-phenylenediamine (DPPD)



p′-(*p*-Toluenesulfonylamido)diphenylamine (TSDP)



N,N′-Di(1,4-dimethylpentyl)-*p*-phenylenediamine (DMPPD)

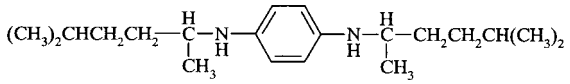


Fig. 1. Chemical Structures of PPD Derivatives

MATERIALS AND METHODS

Reagents —— MHPPD (Mw. 296.46, CAS registry No. 15233-47-3) was a black liquid, and supplied by Seiko Chemical Co., Ltd. (Tokyo, Japan). IPPD, DMBPPD, *N*,*N*′-di-2-naphtyl-*p*-phenylenediamine (DNPD), *N*,*N*′-diphenyl-*p*-phenylenediamine (DPPD), *p*′-(*p*-toluenesulfonylamido)diphenylamine (TSDP), *N*,*N*′-di(1,4-dimethylpentyl)-*p*-phenylenediamine (DMPPD), 6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline (ETMDQ), 2,6-di-*tert*-butyl-4-methylphenol (BHT), stylenated phenol (SP) and 2,5-di-*tert*-butyl-hydroquinone (DBHQ) were provided by Ouchisinko Chemical Industrial Co., Ltd. (Tokyo, Japan). These chemicals were used without further purification. The chemical structures of these PPD derivatives are shown in Fig. 1. Benzyl *n*-butyl phthalate (BBP) and di(2-ethylhexyl)-phthalate (DEHP) were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). All other reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Samples —— Eight samples of "farming" boots from six manufacturers commercially available in Japan were used for the study. The manufacturing date was unknown.

Extraction of Rubber Additives —— Extraction of additives from rubber products was carried out by the method of Kaniwa *et al.*[14] 1.0 g of sample was cut into pieces, 1 mm × 10 mm, and placed in a 50 ml glass centrifuge tube. Then, 10 ml acetone : chloroform (1 : 1) was added to the tube followed by shaking for 30 min at room temperature. The liquid phase was separated by filtration, and collected in a 100 ml round-bottomed flask. The residue was reextracted three times with the same solvent mix-

ture. The extracts were combined, and evaporated in a vacuum rotary evaporator at 50°C.

Column Chromatography —— The extract was dissolved in 1 ml dichloromethane and applied to a glass column (1.0 cm i.d. × 30 cm) packed with 5 g silica–gel (particle size 0.063–0.200 mm, Merck & Co., Inc., Darmstadt, Germany) suspended in hexane. The extract was eluted sequentially with 50 ml diethylether : hexane (5 : 95), diethylether : hexane (10 : 90), diethylether : hexane (20 : 80) and diethylether : hexane (50 : 50). Each eluate was concentrated in a vacuum rotary evaporator, and the final volume was made up to 5 ml or 10 ml using dichloromethane.

HPLC System —— HPLC equipment consisted of an LC-6A pump (Shimadzu Co., Kyoto, Japan), a Rheodyne manual injector fitted with a 200 μl injection loop, a column oven (Model 3510, Senshu Scientific Co., Ltd., Tokyo, Japan), and a UV-VIS detector (Soma optics, Ltd., Tokyo, Japan), or a Shimadzu SPD-M6A spectorophotometric detector. The system was controlled by an NEC 9821Xa16 computer (NEC, Tokyo, Japan), a CBM-10A communications bus module and CLASS-LC10 software (Shimadzu Co., Kyoto, Japan). TSK-GEL ODS-80TS QA (4.6 mm i.d. × 150 mm, Tosoh, Tokyo, Japan) was used as the analytical column and the mobile phase was methanol : water (85 : 15). The flow rate was 1.0 ml/min and the column temperature was 35°C. Detection was carried out at 290 nm. A 10 μl aliquot of the sample solution was injected into the HPLC system and each PPD compound was identified from its retention time.

RESULTS AND DISCUSSION

Calibration

The ultraviolet absorption spectrum of MHPPD in methanol is shown in Fig. 2. Table 1 gives the peak wavelength of eight PPD derivatives. Almost all PPD derivatives had a maximum absorption in the region of 290 nm. Accordingly, a wavelength of 290 nm was selected to determine these PPD derivatives, including MHPPD.

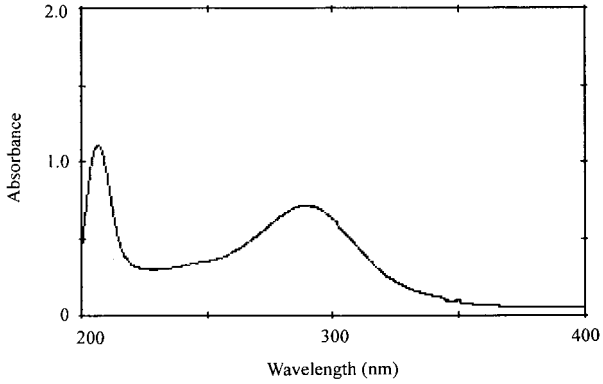


Fig. 2. Ultraviolet Absorption Spectrum of MHPPD in Methanol
MHPPD concentration was 10 μg/ml.

IPPD and DMBPPD are frequently used as rubber antioxidants[5] and so, we chose a mobile phase for HPLC which eluted these PPD and MHPPD within 15 min. Using methanol : water (85 : 15) as the mobile phase, the chromatogram of MHPPD showed two peaks (P1 and P2) at retention times of 9 min and 10.2 min, respectively (Fig. 3). Several PPDs displayed two or three peaks (Figs. 3, 4). We believe that the degree of hydrogen bonding of the –NH of PPDs to the –OH of the ODS column may contribute to the change in peak retention. However, the residual silanol groups were at a minimum in the ODS column used in this study. To prevent peak separation due to this factor, the conversion of PPDs to the derivatives using ion-pair reagents *etc.* seems to be a good idea. However, further exami-

Table 1. Peak Wavelength in the Ultraviolet Absorption Spectrum of Eight PPD Derivatives

Chemical	Abbreviation	Peak wavelength (nm)
N-(1-Methylheptyl)-*N*′-phenyl-*p*-phenylenediamine	MHPPD	290
p-Phenylenediamine	PPD	242
N-Isopropyl-*N*′-phenyl-*p*-phenylenediamine	IPPD	289
N-1,3-Dimethylbutyl-*N*′-phenyl-*p*-phenylenediamine	DMBPPD	289
N,*N*′-Di-2-naphtyl-*p*-phenylenediamine	DNPD	320
N,*N*′-Diphenyl-*p*-phenylenediamine	DPPD	302
p′-(*p*-Toluenesulfonylamido)diphenylamine	TSDP	293
N,*N*′-Di(1,4-dimethylpentyl)-*p*-phenylenediamine	DMPPD	299

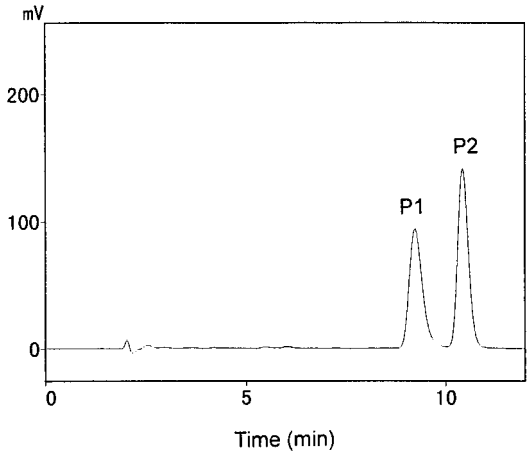


Fig. 3. HPLC Chromatogram of MHPPD

Ten microliters MHPPD (114 μg/ml) in dichloromethane was injected. HPLC conditions: column, ODS (4.6 mm i.d. × 150 mm); column temperature, 35°C; Mobile phase, methanol : water (85 : 15), flow rate, 1.0 ml/min; detection wavelength, 290 nm.

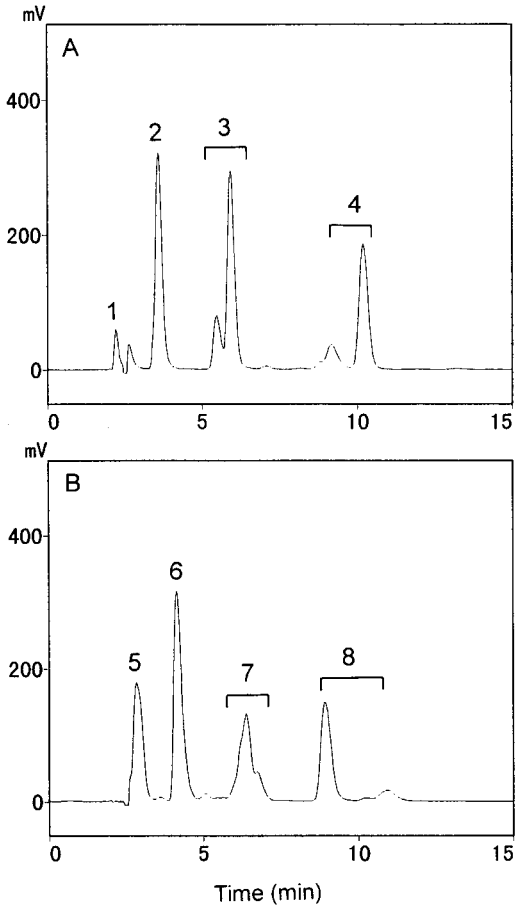


Fig. 4. HPLC Chromatograms of PPD Derivative Standard Mixtures

The concentration of each chemical in dichloromethane was about 100 μg/ml. HPLC conditions were as described in Fig. 3. Mixture A; 1: PPD, 2: IPPD, 3: DMBPPD, 4: MHPPD. Mixture B; 5: TSDP, 6: DPPD, 7: DMPPD, 8: DNPD.

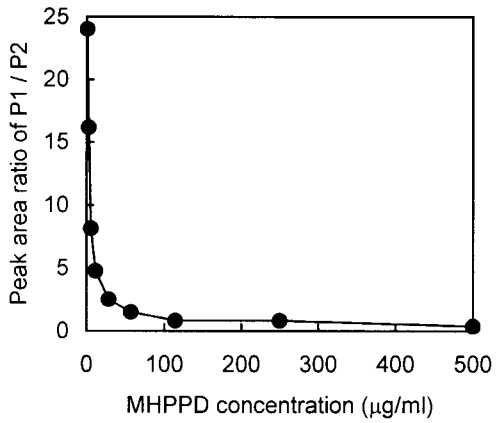


Fig. 5. Peak Area Ratio of P1 and P2 at Each Concentration of MHPPD

nation of the HPLC condition is required. Intramolecular structural change due to long *N*-alkyl groups or interaction between test chemicals and water in the mobile phase may also contribute to the peak separation. Therefore, we injected test chemical solution directly into the HPLC system. Each PPD was well separated under these conditions (Fig. 4).

The area ratio of P1 and P2 varied with the concentration of MHPPD injected (Fig. 5). The P1 area increased with a decrease in concentration and the determination of MHPPD was carried out using total area of P1 and P2. Linear calibration curve for MHPPD was obtained over the range 0.1–500 μg/ml ($\gamma = 0.996$). For DMBPPD and IPPD, calibration curves were obtained over the range 0.1–400 μg/ml. The detection limit of MHPPD, defined as the concentration that produced a signal equal to 3 times the background noise level, was 0.05 μg/ml. The determination limit of MHPPD, DMBPPD and IPPD in samples was 1 μg/g. This sensitivity was sufficient for the determination of chemicals used as rubber antioxidants.

Extraction of MHPPD from Rubber Samples

Because acetone : chloroform (1 : 1) was reported to be a good solvent for extracting various additives, including IPPD, DMPPD, from rubber samples,[14] we chose this to extract MHPPD. The recovery was estimated by addition of 128 μg MHPPD to the rubber sample. The recovery obtained from spiked samples was 93 ± 7% ($n = 4$). The MHPPD in rubber samples was completely extracted by shaking four times with 10 ml acetone : chloroform (1 : 1) for 30 min.

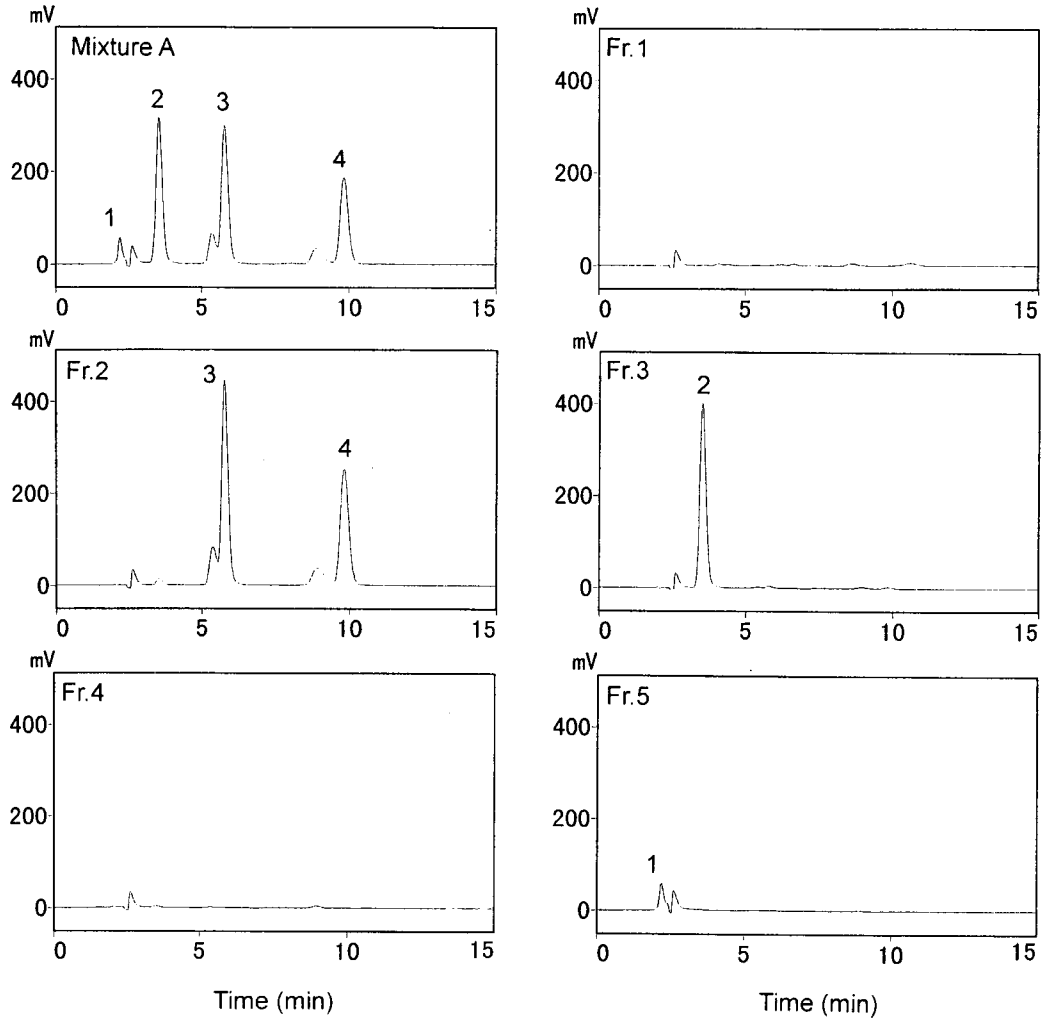


Fig. 6. Elution Pattern of PPD Derivatives from the Silica-Gel Column

Mixture A; 1: PPD, 2: IPPD, 3: DMBPPD, 4: MHPPD. Mixture B; 5: TSDP, 6: DPPD, 7: DMPPD, 8: DNPD. Mixture A or B containing about 100 μg of each chemical was loaded on to a glass column packed with 5 g silica-gel dispersed in hexane, and fractionated with 50 ml diethylether : hexane (5 : 95) (Fr.1), diethylether : hexane (10 : 90) (Fr.2), diethylether : hexane (20 : 80) (Fr.3), diethylether : hexane (50 : 50) (Fr.4) and methanol (Fr.5).

Silica–Gel Column Chromatography

The separation of MHPPD from other rubber additives was carried out using silica–gel column chromatography. MHPPD (5 or 100 μg) was applied to a glass column packed with 5 g silica–gel suspended in hexane, and eluted with 50 ml of a series of diethylether : hexane mixtures. The elution patterns of eight PPDs are shown in Fig. 6. MHPPD was not eluted in the diethylether : hexane (5 : 95) fraction (Fr.1), but was eluted with diethylether : hexane (10 : 90). The recovery of MHPPD following fractionation was $95 \pm 8\%$ ($n = 5$). DMBPPD and DPPD were also eluted in the diethylether : hexane (10 : 90) fraction (Fr.2). IPPD and DNPD were detected in the diethylether : hexane (20 : 80) fraction (Fr.3). The retention time of DNPD was such that it overlapped with the MHPPD peaks in the HPLC chromatogram, but MHPPD and DNPD could be fractionated separately by silica–gel column chromatography.

As far as separation from other types of antioxidants and plasticizers was concerned, DEHP could be detected by reversed phase HPLC with a long retention time and was eluted first by diethylether : hexane (5 : 95). The peak of phenolic antioxidant BHT was close to the first of two MHPPD peaks in the HPLC chromatogram, but this also eluted in Fr.1 (data not shown). In Fr.2, several antioxidants (ETMDQ, SP and DBHQ) and plasticizers BBP were found to elute from the silica–gel column.[6,14] These chemicals did not overlap with MHPPD and other PPD derivatives. Therefore, MHPPD could be determined accurately without interference from other chemicals.

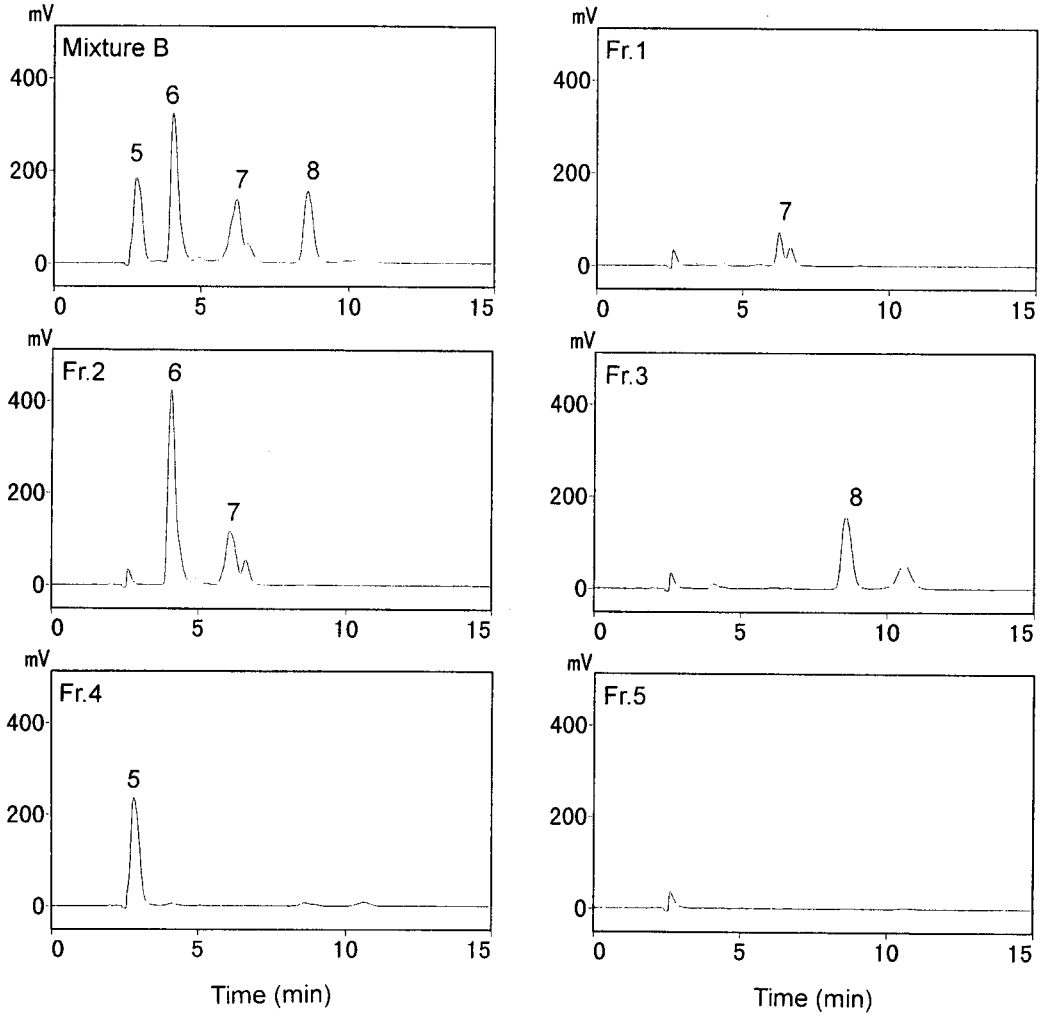


Fig. 6. Continued

Determination of PPD Derivatives in Rubber Boots

The matrix consisted of various rubber additives obtained by extraction and did not dissolve completely in the methanol solution used as the HPLC mobile phase. This insolubility causes an under estimation of rubber additives in samples. In previous studies, the extract of rubber products was dissolved in benzene[6,14] or dichloromethane.[13,17] In this study, the sample solution was prepared using dichloromethane, and injected into the HPLC system. Eight samples of rubber boots were analyzed. Among PPDs, DMBPPD and IPPD were detected, and their content is shown in Table 2. Kaniwa *et al.* detected 177 μg/g IPPD in farmer's rubber boots which led to allergic contact dermatitis.[14] The patients had a positive reaction to IPPD at patch testing, so IPPD was identified as a causative agent.[14] In another case, 95 μg/g DMBPPD was found in rubber boots causing contact dermatitis.[7] Therefore, the amounts of IPPD and DMBPPD found in the rubber boots tested in the present study are sufficient to cause contact dermatitis in patients who are already sensitized. No MHPPD was detected in any

Table 2. Content of PPD Derivatives in Rubber Boots

Sample			Content (μg/g)		
No.	Manufacturer	Color	MHPPD	DMBPPD	IPPD
1	A	Black	n.d.[a)]	1808	n.d.[a)]
2	A	Blue	n.d.	296	126
3	B	Brown	n.d.	n.d.	n.d.
4	B	Black	n.d.	n.d.	n.d.
5	C	Black	n.d.	488	n.d.
6	D	Black	n.d.	n.d.	n.d.
7	E	Black	n.d.	n.d.	886
8	F	Black	n.d.	145	789

a) n.d. = not detected (< 1 μg/g).

of the samples (Table 2). MHPPD is reported to be added to tire sidewalls to make their resistant to abrasion, ozone and water,[10,11] so the use of MHPPD may be limited to this special type of rubber product. To prevent allergic contact dermatitis from rubber products, the manufactures should not use chemicals that have a strong sensitization potential but try to replace them with other, safer, chemicals. The sensitization potential of MHPPD was weaker than that of IPPD and DMBPPD,[12] so MHPPD could be used instead of IPPD or DMBPPD. So, our new method will be useful for monitoring PPD antioxidants (variations in chemical type and content) in rubber products.

REFERENCES

1) The Society of Rubber Industry, Japan (ed.), "Gomu Kogyo Binran 4th ed", Tokyo, pp. 425–443, 1994.
2) Cronin E., "Contact Dermatitis", Churchill, Livingstone, Edinburgh, pp. 714–770, 1980.
3) Magnusson B., Kligman A.M., "Allergic Contact Dermatitis in the Guinea pig", Charles C Thomas, Springfield, IL (1970).
4) Nakamura A., Momma J., Sekiguchi H., Noda T., Yamano T., Kaniwa M., Kojima S., Tsuda M., Kurokawa Y., *Contact Dermatitis*, **31**, 72–85 (1994).
5) Ikarashi Y., Ohno K., Momma J., Tsuchiya T., Nakamura A., *Fd. Chem. Toxicol.*, **32**, 1067–1072 (1994).
6) Kaniwa M., Kojima S., Nakamura A., Ishihara M., *Eisei Kagaku*, **28**, 137–145 (1982).
7) Kaniwa M., Nishioka K., Miyako F., Jidoi J., Isama K., Nakamura A., *Environ. Dermatol.*, **3**, 64–70 (1996).
8) Carlsen L., Anderson K.E., Egsgaard H., *Contact Dermatitis*, **17**, 118–121 (1987).
9) Herve-Bazin B., Gradiski D., Duprat P., Marignac B., Foussereau J., Cavelier C., Bieber P., *Contact Dermatitis*, **3**, 1–15 (1977).
10) Kondo H., Yamada T., *Jpn. Kokai Tokkyo Koho*, JP63010646 (1988).
11) Nishimaki Y., *Jpn. Kokai Tokkyo Koho*, JP11172049 (1999).
12) Shimizu M., Noda T., Abstract of papers, the 36th Annual Meeting of Zenkoku Eisei Kagaku Gijyutu Kyogikai, pp. 190–191, 1999.
13) Kaniwa M., Isama K., Nakamura A., Miyako F., Jidoi J., Nishioka K., *Environ. Dermatol.*, **2**, 170–177 (1995).
14) Kaniwa M., Kojima S., Nakamura A., Kanto H., Ito M., Ishihara M., *Eisei Kagaku*, **30**, 126–137 (1984).
15) Kawamura Y., Miura M., Sugita T., Yamada T., Takeda M., *J. Food Hyg. Soc. Japan*, **37**, 272–280 (1996).
16) Kawamura Y., Miura M., Sugita T., Yamada T., *J. Food Hyg. Soc. Japan*, **38**, 27–33 (1997).
17) Kaniwa M., Kojima S., Nakamura A., Kantoh H., Itoh M., Ishihara M., *Eisei Kagaku*, **32**, 197–211 (1986).