Ⅳ．研究成果の刊行物・別刷り

6．生体機能化されたチタン合金の生物学的安全性評価

国立医薬品食品衛生研究所
松岡厚子、伊佐間和郎

1．はじめに

擬似体液中でアパタイト形成能が高い医用材料は、生体内で骨と直接結合することが期待できる。近年、アパタイト形成能を付与したアルカリ加熱処理チタン合金が製品化され、さらに、より高いアパタイト形成能を付与するためにアルカリ処理したチタン合金へのカルシウム導入も図られている。そこで、我々は、骨組織適合性の高い Ti-Zr-Nb 合金にアルカリ処理後カルシウム導入のための表面処理を施し、そのアパタイト形成能を評価し、加えて、カルシウム導入した Ti-Zr-Nb 合金の細胞毒性試験及び骨芽細胞適合性試験を行って生物学的安全性を評価した。

2．実験

2.1 試験材料及び表面処理

チタン合金として、Ti と Zr の原子比を 1:1 に固定し、Nb 量が異なる Ti-Zr、Ti-Zr-4Nb、Ti-Zr-8Nb、Ti-Zr-16Nb 及び Ti-Zr-24Nb を用いた。また、対照材料として Ti-6Al-4V、純金属として Ti、Zr 及び Nb を用いた。これらの材料を NaOH 溶液を用いてアルカリ処理し、その後、$CaCl_2$ 溶液又は $Ca(OH)_2$ 溶液を用いてカルシウム導入を施した。材料表面のカルシウム導入量は、蛍光 X 線分析法（XRF）により測定した。擬似体液としてカルシウム及びマグネシウムイオンを含有するハンクス平衡塩溶液を用い、表面処理した材料を 37.0℃で 1 週間浸漬した。擬似体液浸漬後、材料表面に形成したアパタイトを、走査型電子顕微鏡（SEM）により観察し、フーリエ変換赤外光音響分光分析法（FT-IR/PAS）により定量的に解析した。

2.2 細胞毒性試験

医療機器の生物学的安全性評価のための試験法に従い、50 個のチャイニーズ・ハムスター肺由来線維芽細胞株 V79 細胞を材料ディスク（直径 14.0 mm、厚さ 1.0 mm）の上に播種し、10%牛胎児血清添加イーグル MEM 培地で 6 日間培養した。その後、ギムザ染色して形成したコロニー数を数え、コントロール群のコロニー数に対する割合（コロニー形成率）を算出した。

2.3 骨芽細胞適合性試験

正常ヒト骨芽細胞（NHOst, Cambrex CC-2538）を材料ディスクの上に播種し、5 mM β-グリセロリン酸ナトリウム及び 10% 牛胎児血清添加 α-MEM 培地で 2 週間培養した。その後、細胞増殖の指標として、WST-8 法により相対細胞数を測定した。さらに、細胞分化の指標として、パ

ラニトロフェニルリン酸を基質に用いて骨芽細胞のアルカリホスファターゼ（ALP）活性を測定した。

3．結果及び考察

3.1 カルシウム導入量

Ti-Zr、Ti-Zr-4Nb、Ti-Zr-8Nb、Ti-Zr-16Nb、Ti-Zr-24Nb、Ti-6Al-4V 及び Ti は、アルカリ処理後 $CaCl_2$ 処理に比べて、アルカリ処理後 $Ca(OH)_2$ 処理の方がカルシウム導入量が約 2 倍高かった。また、Zr は、アルカリ処理後 $CaCl_2$ 処理ではカルシウムを導入できなかったが、アルカリ処理後 $Ca(OH)_2$ 処理ではカルシウムを導入できた。また、Nb はどちらの処理法でもカルシウムを導入できなかった。

3.2 アパタイト形成能

Nb 含量にかかわらず未処理の Ti-Zr-Nb 合金は、1 週間の擬似体液浸漬によりアパタイトを形成しなかった。一方、Ti-Zr 及び Ti-Zr-4Nb は、アルカリ処理、アルカリ処理後 $CaCl_2$ 処理及びアルカリ処理後 $Ca(OH)_2$ 処理によって、アパタイトを形成した（図 1）。これらのアパタイト形成能は、アルカリ処理 < アルカリ処理後 $CaCl_2$ 処理 < アルカリ処理後 $Ca(OH)_2$ 処理の順に高くなった。さらに、Ti-Zr-8Nb は、アルカリ処理及びアルカリ処理後 $CaCl_2$ 処理ではアパタイトを形成しなかったが、アルカリ処理後 $Ca(OH)_2$ 処理によってアパタイトを形成した（図 1）。また、Ti-Zr-16Nb 及び Ti-Zr-24Nb は、いずれの表面処理でもアパタイトを形成しなかった。材料表面へのカルシウム導入量が高かった Ti-Zr-Nb 合金は、擬似体液浸漬によるアパタイト形成能も高かった。

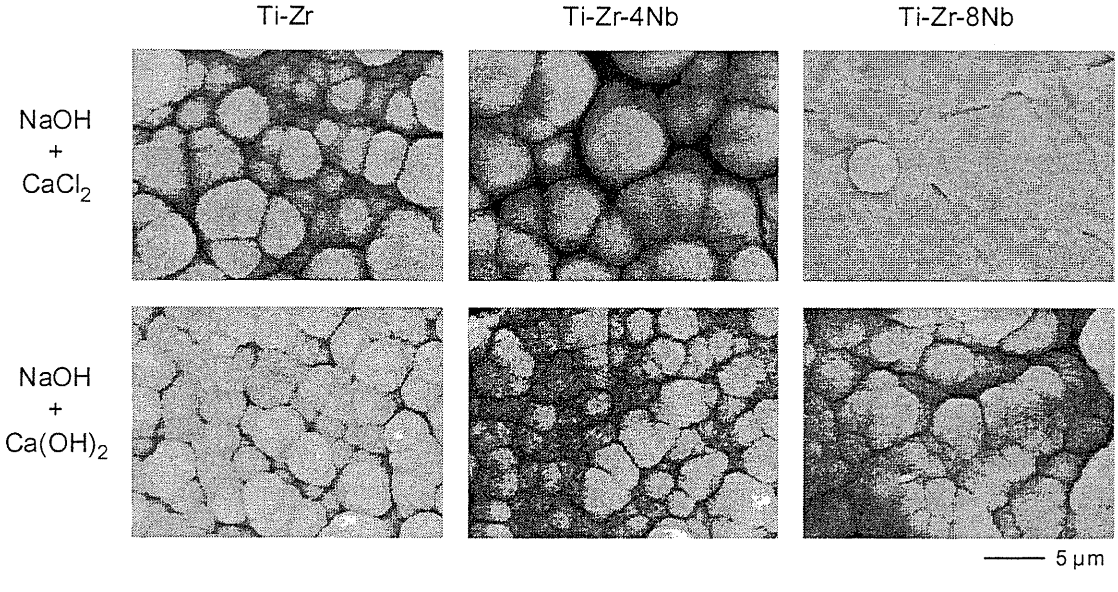


図 1　NaOH+$CaCl_2$ 処理及び NaOH+$Ca(OH)_2$ 処理した Ti-Zr、Ti-Zr-4Nb 及び Ti-Zr-8Nb のハンクス平衡塩溶液浸漬後の SEM 観察像

3.3 細胞毒性

未処理の Ti-6Al-4V は弱い細胞毒性を示したが、表面処理によって細胞毒性は問題のないレベルになった。その他の材料は、未処理及びいずれの処理においても、細胞毒性は認められなかった（図2)。

3.4 骨芽細胞適合性

骨芽細胞の細胞数は、未処理の Ti-6Al-4V と比べて、アルカリ処理、アルカリ処理後 $CaCl_2$ 処理及びアルカリ処理後 $Ca(OH)_2$ 処理を施した Ti-6Al-4V ではいずれも増加した。その他の材料では、未処理及びいずれの処理においても骨芽細胞の細胞数に相違は認められなかった。骨芽細胞の ALP 活性は、Ti-Zr、Ti-Zr-4Nb、Ti-6Al-4V 及び Ti では、未処理、アルカリ処理、アルカリ処理後 $CaCl_2$ 処理及びアルカリ処理後 $Ca(OH)_2$ 処理の順に増加傾向が認められた。その他の材料では、未処理の Ti-6Al-4V と比べて ALP 活性は高かったものの、処理の有無による ALP 活性の相違は認められなかった。

アルカリ処理後 $Ca(OH)_2$ 処理は、Ti や Ti-Zr-Nb 合金等に高いアパタイト形成能を付与することができ、さらに、本研究で骨芽細胞の分化を促進させることが分った。Ti-Zr-Nb 合金は力学的性質にも特長があり、有効性及び安全性の高い金属材料として、埋植医療機への応用が期待できる。

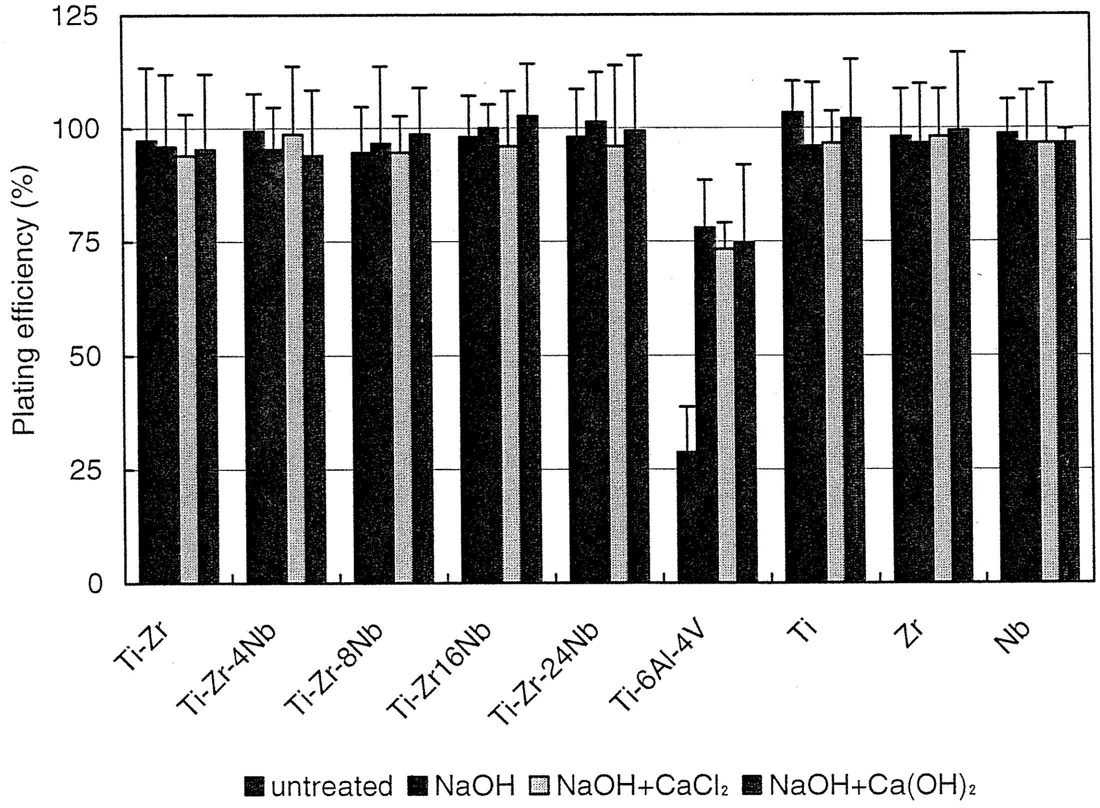


図2　表面処理した材料のコロニー形成率

— Regular Article —

Determination of Dimethyl Fumarate and Other Fumaric and Maleic Acid Diesters in Desiccants and Consumer Products in Japan

Tsuyoshi Kawakami,[*, a] **Kazuo Isama,**[a] **Atsuko Matsuoka,**[b] **and Tetsuji Nishimura**[a]

[a]*Division of Environmental Chemistry and* [b]*Division of Medical Devices, National Institute of Health Sciences, 1–18–1 Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan*

(Received December 14, 2010; Accepted February 8, 2011; Published online February 18, 2011)

Recently, many contact dermatitis cases related to leather furniture and footwear containing dimethyl fumarate (DMF) as an anti-mold agent have been reported in European countries. We investigated the concentrations of DMF and several fumaric and maleic acid diesters in desiccants and household products (footwear and rack) enclosed with a desiccant sachet in Japan. We sorted the product samples by material, and analyzed the product parts that can come into contact with the skin of consumers. Twenty-one desiccant samples and eighteen product samples (seven footwear products and one rack product) were analyzed. DMF was detected in the range of 0.11–2.3 mg/kg in two desiccant samples and three product samples (different parts of one product). The DMF concentrations detected in this study exceeded the value regulated by the European Union (0.1 mg/kg); the concentration of one desiccant sample was exceeded 1.0 mg/kg which showed a strong reaction in the patch tests in a precious study. The notes printed on the sachets of the desiccant samples containing DMF read "mold-proof desiccant" and "do not eat" in one case and merely "do not eat" in the other case. DMF has strong sensitization and irritation activities; hence, it is necessary to analyze more samples to prevent DMF-related contact dermatitis in Japan. Dibutyl maleate (DBM) was detected in the rack product and enclosed desiccant; its concentration ranged from 29 to 720 mg/kg. DBM may be a constituent of the adhesive used for the rack. Further investigation is necessary to verify the cross-reaction of DBM with DMF.

Key words —— dimethyl fumarate, contact dermatitis, desiccant, footwear, mold-proof

INTRODUCTION

Dimethyl fumarate (DMF) is a white crystalline powder that undergoes sublimation at room temperature. DMF has been known as an inhibitor of mold growth and an antibacterial substance.[1, 2] In addition, DMF has been used as the essential pharmaceutical component in the oral treatment of psoriasis (Fumaderm®) in Germany since 1994.[3] On the other hand, DMF is cytotoxic (epidermoid cell line A431, LD_{50}: 5.04 µg/ml), and it induces non-immunological contact urticaria and allergic contact dermatitis.[4]

Since the summer of 2006, many dermatitis cases related to leather furniture such as sofas and armchairs have been reported in European countries, especially in the U.K. and Finland.[5, 6] At first, the causative substance was not identified; however the common factor in all cases was the use of leather furniture manufactured in China. In 2008, it was reported that DMF was detected in the sofas and armchairs used by the patients with contact dermatitis, and DMF was identified as the causative substance of the contact dermatitis caused by Chinese furniture.[7] After this identification of the causative substance, many cases caused by DMF have been reported; these cases are attributed not only to leather furniture but also to other consumer products such as footwear[8, 9] and clothing.[10] DMF was frequently used in the desiccant sachets placed inside furniture and enclosed in footwear boxes. Evaporated DMF impregnated the products, thereby protecting them from mold. However, consumers were

---

[*] To whom correspondence should be addressed: Division of Environmental Chemistry, 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: tkawa@nihs.go.jp

adversely affected when they came into contact with the products.

DMF has been banned in products manufactured in the European Union (EU), according to the Biocide Directive (EU Directive 98/8/EC).[11] However, non-EU countries may continue using DMF as a biocide to prevent mold growth during the transport and storage of products. Since May 1, 2009, the European commission banned DMF in consumer products in the EU market (EU Directive 2009/251/EC).[12] This European directive required a DMF concentration of less than 0.1 mg/kg of the product or product part. This regulated value was considered to be sufficiently lower than the concentration (1.0 mg/kg) that showed a strong reaction in the patch test.[7] However, the number of contravention cases reported by Rapid Alert System for non-food consumer products (RAPEX),[13] which reports weekly violations of EU regulations in the EU market, exceeded 100 from May 1, 2009, to April 30, 2010. The cases related to footwear products accounted for more than 90% of all the cases.

On the other hand, to the best of our knowledge, cases of contact dermatitis related to DMF were not reported in non-EU countries, except for one case in Canada.[14] Although it is thought that similar products with sachets containing DMF are distributed in the Japanese consumer product market, contact dermatitis cases related to DMF and the amounts of DMF in desiccants and household products have not been investigated in Japan. Therefore, we investigated the concentrations of DMF in desiccants and several household products in Japan.

Furthermore, compounds having a chemical structure similar to that of DMF, such as fumaric acid diesters, maleic acid diesters, and acryl acid esters, induce contact dermatitis.[15–17] These compounds cause skin irritation and sensitization, and they also induce a cross-reaction with DMF. Therefore, in this study, we also determined the concentrations of diethyl fumarate (DEF), dibutyl fumarate (DBF), dimethyl maleate (DMM), diethyl maleate (DEM), and dibutyl maleate (DBM) in desiccants and several household products.

MATERIALS AND METHODS

Samples —— Sachets containing a desiccant were provided by volunteers who purchased footwear, bags, racks, *etc.* from retail stores in Japan. In addition, footwear products (with a desiccant sachet) were purchased from several retail stores in Japan from June to July 2010. The details of the desiccant samples and product samples are shown in Tables 1 and 2, respectively. All the desiccant samples were silica gel, except for S9 (a clay-type desiccant, Table 1). The product samples were sorted by material, and the product parts that can come into contact with the skin surface were analyzed, except for the mount paper of the rack sample (P10-3). A total of 21 desiccant samples and 18 product samples (seven footwear products and one rack product) were analyzed.

Materials —— The household products analysis grade of DMF and chemical analysis grade of DEF were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Chemical analysis grade of DBF, DMM, DEM, and DBM were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). An environmental analysis grade of naphthalene-d_8 was obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). Pesticide residue grade of ethyl acetate and methanol were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). Silica gel powder (Silica Gel 60, particle size: 0.040–0.063 mm) used as a blank sample was obtained from Merck (Darmstadt, Germany).

Sample Processing —— The desiccant sample was crushed by an agate mortar, and 0.5 g of the sample was placed into a glass tube with 5 ml of ethyl acetate. Then, ultrasonic extraction was performed for 5 min, and the tube was centrifuged for 2 min (3000 rpm). After centrifugation, the supernatant was obtained. This extraction procedure was conducted twice. The supernatants were combined and concentrated to approximately 1 ml with a rotary evaporator while maintaining the temperature of the water bath below 40°C. Next, the solution was concentrated to below 0.5 ml by a gentle N_2 stream. Twenty-five micro liters of ethyl acetate solution containing 1 µg/ml of naphtalane-d_8 as an internal standard was added, and the sample volume was adjusted to 0.5 ml. This solution was then analyzed by GC/MS.

The product sample was cut, and 0.5 g of the sample was placed into a glass tube with 20 ml of methanol. Then, this tube was shaken for 10 min, and ultrasonic extraction was performed for 5 min. After extraction, the solution was filtered; the residue was washed with about 10 ml of methanol and the washing combined with the filtrate. The sample solution was concentrated to approximately 2 ml with a rotary evaporator while maintaining the

Table 1. List of the Desiccant Samples Studied

Sample name	The product enclosed with the desiccant sachet	Country	The time obtained the sachet	How to obtain[a]	Remarks
S1	Sports shoes	China	Mar-09	V	The sachet stayed for long time under room temperature.
S2	Ink toner	Unknown	Mar-09	V	Laser color printer
S3	Leather bag	Vietnam	Mar-09	V	The sachet stayed for long time under room temperature.
S4	Children sofa	China	Sep-09	V	Synthetic leather
S5	Ornament for the new year	Japan	Dec-09	V	
S6	Sandals	China	May-10	P	"Do not eat" and "mold-proof desiccant" were printed on the sachet in Japanese, English, and Chinese.
S7	Leather bag	China	May-10	V	Tote bag for woman, synthetic leather
S8	Belt	China	May-10	V	Synthetic leather
S9	Sofa	China	May-10	V	Clay type desiccant
S10	Rack	China	May-10	V	Synthetic leather with mount paper
S11-1 S11-2	Sports shoes	China	Jun-10	P	"Do not eat" and "mold-proof desiccant" were printed on the sachet in Japanese, English, and Chinese. The two sachet with same notes were enclosed together.
S12	Shoes	Unknown	Jun-10	V	The detail of products was unknown
S13	Shoes	Unknown	Jun-10	V	The detail of products was unknown
S14	Shoes	Unknown	Jun-10	V	The detail of products was unknown
S15	Shoes	Unknown	Jun-10	V	The detail of products was unknown
S16	Shoes	China	Jul-10	P	Synthetic leather and fiber
S17	Sandals	China	Jul-10	P	Synthetic leather, sandal for woman
S18	Shoes	Unknown	Jul-10	P	Synthetic leather and fiber
S19	Sandals	China	Jul-10	P	Synthetic leather
S20-1 S20-2	Sports shoes	China	Jun-10	P	"Do not eat" was printed on the sachet in English, French, Dutch, and German. However, the notes of these sachets were written by different font type.

a) V: provided from volunteer, P: purchased from retail store.

temperature of the water bath below 40°C. The sample solution was passed through a graphite carbon cartridge (InertSep GC 300 mg/6 ml, GL Science, Tokyo, Japan) washed with 4 ml of ethyl acetate and 4 ml of methanol before sample loading. Next, 2 ml of methanol containing 50% ethyl acetate was passed through the cartridge twice, and 6 ml of eluate was concentrated to approximately 1 ml with a rotary evaporator while maintaining the temperature of the water bath below 40°C. Then, to change the dominant solvent, 5 ml of ethyl acetate added to sample solution and concentrated to 2 ml with a rotary evaporator while maintaining the temperature of the water bath below 40°C. The sample solution was passed through a neutral alumina cartridge (InertSep AL-N 1 g/6 ml, GL Science) washed with 4 ml of ethyl acetate before sample loading. Next, 2 ml of ethyl acetate was passed through the cartridge and 4 ml of eluate was concentrated to below 0.5 ml by a gentle N_2 stream. Twenty-five micro liters of ethyl acetate solution containing 1 μg/ml of naphtalane-d_8 as an internal standard was added, and the sample volume was adjusted to 0.5 ml. This solution was then analyzed by GC/MS.

GC/MS Analysis —— All the samples in this study were analyzed using a Focus GC with a DSQII MS (Thermo Fisher Scientific, Waltham, MA, U.S.A.). A VF-5 ms fused silica capillary column (length: 30 m, internal diameter: 0.25 mm, film thickness: 0.25 μm, Varian-Agilent, Santa Clara, CA, U.S.A.) was used. The carrier gas used was He with a flow rate of 1.0 ml/min. The temperatures of the injec-

Table 2. List of the Product Samples Studied[a]

Type of product[b]	Sample name	Remarks
Sandals (S6)	P6-1	Sole surface
	P6-2	Mesh cloth strap inside
	P6-3	Synthetic leather strap inside
Rack (S10)	P10-1	Synthetic leather (outside)
	P10-2	Synthetic leather (inside)
	P10-3	Mount paper bonded to synthetic leather (inside)
Sports shoes (S11)	P11-1	Mesh cloth that covers the inside of shoe
	P11-2	Insole surface
Shoes (S16)	P16-1	Mesh cloth that covers the inside of shoe
	P16-2	Insole surface
Sandals (S17)	P17-1	Sole surface
	P17-2	Inside of strap
	P17-3	Strap lining of heel
Shoes (S18)	P18	Insole surface
Sandals (S19)	P19-1	Sole surface
	P19-2	Inside of strap
Sports shoes (S20)	P20-1	Mesh cloth that covers the inside of shoe
	P20-2	Insole surface

a) The sample was obtained the product part that can come into contact with skin, except for P10-3. *b)* The sample name in parenthesis corresponded to Table 1.

Table 3. GC Retention Times, Quantifying and Qualifying Ions, Recovries and its Coeficients of Variation (C.V.%, $n = 3$) and LOD[a] and LOQ[b] of the Compounds Studied

Compound	Retention time (min)	Quantifying ion (m/z)	Qualifying ion (m/z)	Desiccant sample				Product sample			
				Recovery (%)	C.V.(%)	LOD	LOQ	Recovery (%)	C.V.(%)	LOD	LOQ
DMF	5.92	113	59, 85	77	1.1	0.0032	0.012	54	1.3	0.0058	0.012
DEF	7.20	127	99, 126	77	2.0	0.0031	0.0097	87	3.2	0.0060	0.017
DBF	9.94	117	99, 156, 177	73	1.0	0.0096	0.0098	56	3.8	0.017	0.45
DMM	5.88	113	59, 85	62	2.6	0.00058	0.011	40	2.3	0.0020	0.021
DEM	7.06	99	126, 127	91	2.2	0.00087	0.010	61	2.5	0.0013	0.012
DBM	9.64	117	99, 156, 177	77	0.21	0.011	0.012	60	3.6	0.027	0.36
Naphthalene-d_8	7.43	136									

a) LOD (mg/kg): (3.3 × standard deviation)/(slope of calibration curve × relative sensitivity) ($n = 3$). *b)* LOQ (mg/kg): 10 × standard deviation ($n = 3$).

tor, transfer line, and ion source were 250, 280, and 250°C, respectively. The sample was injected in the splitless mode, and the injected volume was 1 µl. The GC oven temperature was initially maintained at 40°C for 0.5 min and the temperature increased to 310°C at a rate of 20°C/min. The oven temperature was then maintained at 310°C for 10 min. The MS was operated in the electron ionization (EI) mode at 70 eV, and the analysis was performed using the selected ion monitoring (SIM) mode. The retention times and the quantifying and qualifying ions are listed in Table 3.

Relatively small amounts of DEF and DBF were observed on the GC/MS chromatogram of DEM and DBM, respectively. We assumed that the DEF and DBF observed on the GC/MS chromatogram were impurities of DEM and DBM or that they were generated by photo-translation under room light or heat-translation in the GC injector; however, the ratios of DEF/DEM and DBF/DBM were almost constant after prolonged exposure to room light or injection at the various temperatures of the GC injector. Therefore, we thought that the concentrations of these compounds could be determined by the sample processing described above and GC/MS conditions adopted in this study. The standard curves of fumaric acid diesters and maleic acid diesters were prepared separately.

RESULTS AND DISCUSSION

Examination of Sample Preparation

First, the effect of N_2 dryness on behavior of DMF was examined, and the recovery of DMF after N_2 dryness was 11% (coefficient of variation: C.V. = 36%, n = 3). Thus, we performed the sample preparation process to prevent evaporation to dryness. Since acetone and ethyl acetate were used as extraction solvents for desiccant samples in previous studies,[18–20] the recoveries of DMF, DEF, and DBF extracted by acetone and ethyl acetate were examined (added 0.1 mg/kg, n = 3). The recoveries using acetone were 78–82% (C.V. = 2.4–9.1%), and those of ethyl acetate were 73–77% (C.V. = 1.0–2.0%). In this study, because reproducibility was considered to be of greater importance, ethyl acetate was selected for as the extraction solvent for the desiccant samples.

Although DMF was extracted from components of shoe samples by ultrasonic extraction using ethyl acetate in a previous study,[8] ethyl acetate could not be used as the extraction solvent in this study because the extracted solution had high viscosity, preventing next sample processing and GC/MS analysis. A high-viscosity solution may have been obtained because of the elution of adhesive and resin components from the product samples. It was observed that the solution extracted from product samples using methanol was not highly viscous; hence, methanol was used as the extraction solvent for product samples in this study. The sample solution was purified by a graphite carbon cartridge because several extracted solutions were colored and muddy. Pure methanol, methanol containing 25% ethyl acetate, and methanol containing 50% ethyl acetate were examined as eluted solutions from the cartridge for DMF, DEF, and DBF (Fig. 1). The results indicated that methanol containing 50% ethyl acetate was the most suitable solution for elution. Although several sample solutions remained colored, further purification was performed using a neutral alumina cartridge that has been reported to purify the extract from leather products.[21] Although the concentration of DMF was determined without interference from monitoring ion on the mass chromatogram by these purification processes, the ions causing interference for DBF and DBM could not be removed completely.

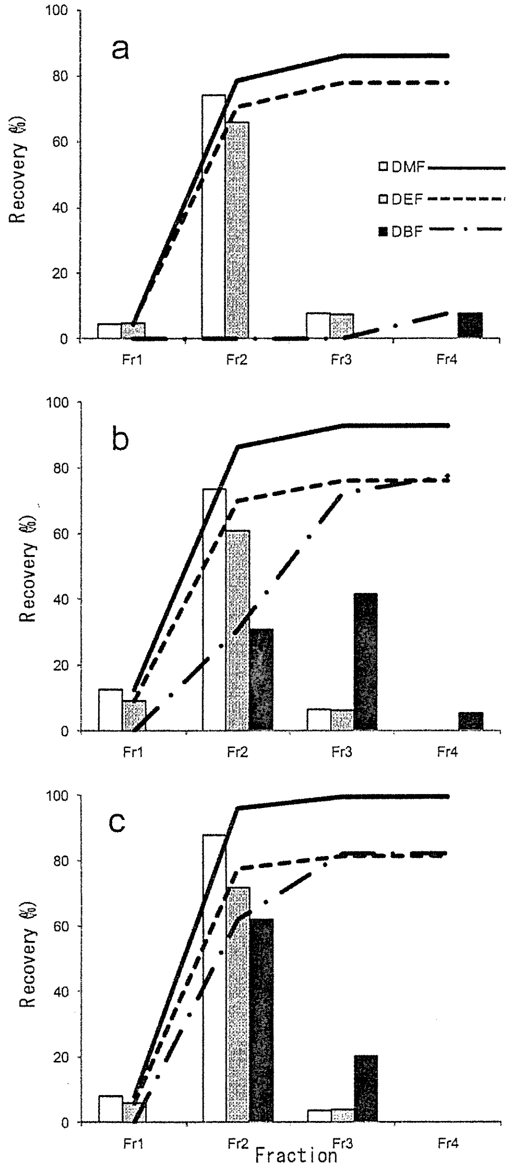


Fig. 1. Effects of Ethyl Acetate Content in Methanol on Elution Pattern of DMF, DEF, and DBF from Graphite Carbon Cartridge

a: 100% methanol, b: methanol containing 25% ethyl acetate, c: methanol containing 50% ethyl acetate. Fr1: eluted sample solution, Fr2: eluted solution 0–2 ml, Fr3: eluted solution 2–4 ml, Fr4: eluted solution 4–6 ml; the lines on the graph represent the accumulated amount of target compound eluted.

Recoveries, Limits of Detection (LOD), and Limits of Quantification (LOQ)

Recovery tests were performed by adding every compound into the samples. For the desiccant samples, 0.05 μg of every compound was added to 0.5 g of a blank sample (n = 3, 0.1 mg/kg). For the product samples, 0.05 μg of every compound was added to 0.5 g of a blank sample that did not contain the chemicals being studied (n = 3, 0.1 mg/kg), except for DBF and DBM because of the reasons described above (0.5 μg of DBF and DBM were added to the blank samples, 1.0 mg/kg, n = 3). The results of the recovery tests are shown in Table 3. The recoveries of DMF were 77% (C.V. = 1.1%) from the desiccant samples and 54% (C.V. = 1.3%) from the product samples. The DMF recovery from the product samples was lower than that from desiccant samples due to evaporation during the several concentration processes involved in purification. The recoveries of the other compounds ranged from 62% to 91% (C.V. = 0.21–2.6%) from desiccant samples and 40–87% (C.V. = 2.3–3.8%) from product samples. Although the recoveries were slightly low, all the C.V. values were below 4%, and it was thought that the reproducibility of the methods in this study was sufficient to determine the concentrations of DMF and other chemicals in the desiccant and product samples. The data obtained in this study were not corrected by the obtained recovery results.

The LOD and LOQ were calculated from the results of recovery tests that involved 0.025 μg being added to 0.5 g of the samples (n = 3, 0.05 mg/kg). In the case of DBF and DBM in the product samples, 0.25 μg of every compound was added. LOD[22] and LOQ[23] were calculated as follows:

$$\mathrm{LOD} = 3.3 \times \rho / ar \qquad (1)$$

$$\mathrm{LOQ} = 10\rho \qquad (2)$$

where ρ is the standard deviation obtained from the results of a low-concentration analysis, a is the slope of the calibration curve, and r is the relative sensitivity. The resulting LOD and LOQ values are listed in Table 3. The LOD and LOQ of DMF were 0.0032 and 0.012 mg/kg in the desiccant samples and 0.0058 and 0.012 mg/kg in the product samples, respectively. The LOQs of DMF obtained in this study were significantly lower than the value mandated by the EU (below 0.1 mg/kg).

Table 4. Concentrations of Fumaric and Maleic Acid Diesters in the Samples

Sample name	DMF	DEF	DBF	DMM	DEM	DBM
S1	—	—	—	—	—	—
S2	—	—	—	—	—	—
S3	—	—	—	—	—	—
S4	—	—	—	—	—	—
S5	—	—	—	—	—	—
S6	2.3	—	—	—	—	—
S7	—	—	—	—	—	—
S8	—	—	—	—	—	—
S9	—	—	—	—	—	—
S10	—	—	8.4	—	—	720
S11-1	—	—	—	—	—	—
S11-2	—	—	—	—	—	—
S12	—	—	—	—	—	—
S13	—	—	—	—	—	—
S14	—	—	—	—	—	—
S15	—	—	—	—	—	—
S16	—	—	—	—	—	—
S17	—	—	—	—	—	—
S18	—	—	—	—	—	—
S19	—	—	—	—	—	—
S20-1	—	—	—	—	—	—
S20-2	0.60	—	—	—	—	—
P6-1	0.21	—	—	—	—	—
P6-2	0.11	—	—	—	—	—
P6-3	0.14	—	—	—	—	—
P10-1	—	—	—	—	—	29
P10-2	—	—	9.6	—	—	340
P10-3	—	—	12	—	—	440
P11-1	—	—	—	—	—	—
P11-2	—	—	—	—	—	—
P16-1	—	—	—	—	—	—
P16-2	—	—	—	—	—	—
P17-1	—	—	—	—	—	—
P17-2	—	—	—	—	—	—
P17-3	—	—	—	—	—	—
P18	—	—	—	—	—	—
P19-1	—	—	—	—	—	—
P19-2	—	—	—	—	—	—
P20-1	—	—	—	—	—	—
P20-2	—	—	—	—	—	—

unit: mg/kg. — Not detected.

Concentrations of DMF in the Samples

The concentrations of DMF in the samples are shown in Table 4. DMF was detected in two desiccant samples, S6 and S20-2, and the corresponding concentrations were 2.3 and 0.60 mg/kg, respectively. The mass chromatogram (m/z = 113) of S6 and the mass spectrum (scan mode: m/z = 50–350) of the DMF detected in S6 are shown in Fig. 2. The S6 and S20-2 sachets were enclosed with sandals (P6) and sports shoes (P20), and these footwear products were manufactured in China. In the case of S6, the notes were printed on the sachet in Chinese, English, and Japanese (Fig. 3a). The notes state that the sachet contains a desiccant with an anti-mold agent that is inedible. Although the same notes were printed on the sachets of S11-1 and S11-2, DMF was not detected in these desiccant samples. In contrast, the notes on the sachet of S20-2 were printed in four languages as follows: "DO NOT EAT" (En-

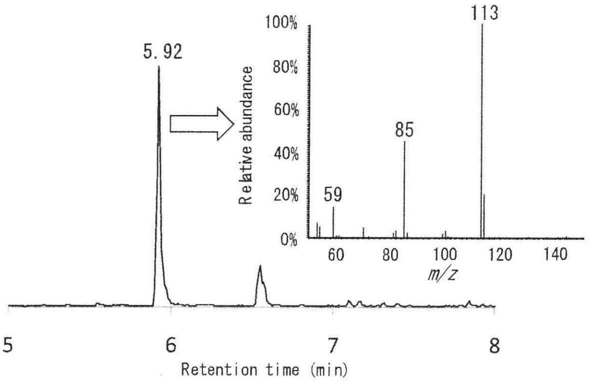
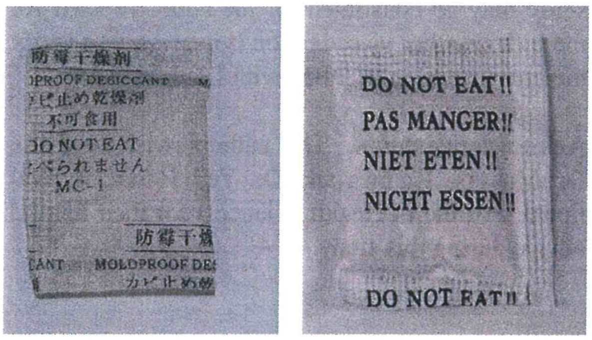


Fig. 2. Mass Chromatogram of DMF Obtained from the Desiccant Sample (S6, selected ion monitoring mode: m/z = 113) and Mass Spectrum of DMF Obtained by Scan Analysis (scan mode: m/z = 50–350).



(a) (b)

Fig. 3. Photographs of Sachets that DMF Was Detected
a: S6, b: S20–2.

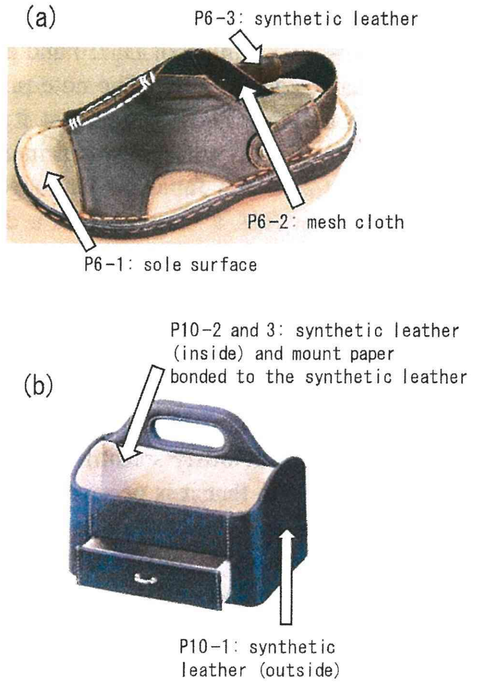


Fig. 4. Photographs of Sandal (a: P6) and Rack (b: P10) that DMF Was Detected

glish), "PAS MANGER" (French), "NIET ETEN" (Dutch), and "NICHT ESSEN" (German) (Fig. 3b); however, no note was printed in Japanese. The same notes were also printed on the sachet of S20-1; however, the font type of the note was different from that used for S20-2, and DMF was not detected in S20-1.

On the other hand, in the case of the sandal product sample (P6), for which DMF was detected in the enclosed desiccant sample, DMF was detected in the three product part samples which were the sole surface (P6-1), the mesh cloth strap inside (P6-2), and the inside of the synthetic leather strap (P6-3) (Fig. 4a). The concentrations of DMF in P6-1, 2, and 3 were 0.21, 0.11, and 0.14 mg/kg, respectively (Table 4). Although DMF was detected in the desiccant sample (S20-2), it was not detected in the product samples of sports shoes (P20-1 and 2) enclosed with the sachet (S20-2). Furthermore, DMF was not detected in any other samples. The DMF concentrations detected in this study exceeded the EU regulated value (0.1 mg/kg), and the concentration of one desiccant sample (S6) was exceeded 1.0 mg/kg which showed a strong reaction in the patch tests.7)

The detection of DMF was expected in S11-1, S11-2, S20-1, P20-1, and P20-2; however, DMF was not detected in these samples. It has been reported that DMF concentration decreases to around one-tenth after two weeks of storage at room temperature without any wrapping.24) It was indicated that DMF was released from such products via evaporation, and it adsorbed on other product.10) Thus, the low concentrations of DMF observed in this study may be attributed to the evaporation of DMF because the desiccant and/or product were produced a long time ago. Furthermore, a small amount of DMF may be used in the desiccants and/or products distributed in the Japanese market. Information regarding the samples investigated in this study was insufficient; hence, we cloud not conclude whether the DMF detected in this study was used intentionally.

In Spain, 37 sachets enclosed with clothes, accessories, footwear, and furniture were collected, and the desiccants contained in the sachets were analyzed by GC/MS.19) It was reported that the concentrations of DMF detected in 27 samples were

0.239 to 2640 mg/kg (more than half of these samples contained over 100 mg/kg of DMF) and a certain sachet contained DMF only. The note printed on the sachet containing DMF stated that it only contains silica gel.[19] "Do not eat" was printed on the sachet of S20-2 in non-Japanese languages in this study. Thus, it was a concern that a desiccant sachet containing DMF enclosed with products distributed in the Japanese market may not have a label stating "mold-proof agent." Furthermore, in EU countries, the detection frequencies of DMF increase in the winter[13] and contact dermatitis related to DMF is induced at very low concentrations if a person has been previously sensitized to DMF. Therefore, we conclude that it is necessary to analyze more samples to prevent contact dermatitis related to DMF in Japan.

Concentrations of Other Compounds in the Samples

DBM was detected in the desiccant sample (S10), and its concentration was very high (720 mg/kg). Furthermore, DBM was detected in the three product samples that were parts of synthetic leather located on the outside and inside surfaces of rack (P10-1 and 2) and mount paper (P10-3) bonded to the synthetic leather inside the rack (Fig. 4b). DBM concentrations of P10-1, 2, and 3 were 29, 340, and 440 mg/kg, respectively. DBF was also detected in S10, P10-2, and 3, respectively. An adhesive containing DBM may be used for bonding synthetic leather and mount paper; hence, DBM may evaporate from the adhesive, and it may be adsorbed on the surface of the synthetic leather and the desiccant. Although DBF may be generated from DBM, the generation process has not been determined thus far. DEF, DMM, and DEM were not detected in any of the samples; this result is similar to the results of a previous study on dermatitis related to furniture containing DMF.[25] Occupational contact dermatitis related to DBM at a factory using adhesives was reported,[16] and the cross-reaction of DBM and DMF is unknown; hence, it is necessary to investigate the cross-reaction of DMF with DBM.

In conclusion, desiccants in the sachets and products (footwear and rack) enclosed with the desiccant sachets were analyzed to determine the concentrations of DMF and several fumaric and maleic acid diesters (DEF, DBF, DMM, DEM, and DBM). The product samples were sorted by material. A total of 21 desiccant samples and 18 product samples (seven footwear products and one rack product) were analyzed. DMF was detected in the range of 0.11–2.3 mg/kg in the two desiccant samples and three product samples (from one product). The DMF concentrations detected in this study exceeded the EU regulated value (0.1 mg/kg), and the concentration of one desiccant sample (S6) was exceeded 1.0 mg/kg which showed a strong reaction in the patch tests in a previous study. The note printed on one of the sachet containing DMF read "mold-proof desiccant, do not eat"; in contrast, the note on the other sachet merely read "do not eat." DMF has strong sensitization and irritation activities; hence, it is necessary to analyze more samples to prevent contact dermatitis related to DMF in Japan. DBM was detected in the rack product and the desiccant enclosed with this rack; its concentration ranged from 29 to 720 mg/kg. The DBM detected in this study may be constituent of the adhesive used for the rack. Further investigation is necessary to verify the cross-reaction of DBM with DMF.

Acknowledgements The authors wish to thank Dr. Masaaki Kaniwa and Dr. Yuji Haishima (National Institute of Health Sciences) for their helpful advice during this study.

REFERENCES

1) Islam, N. M. (1982) Inhibition of mold in bread by dimethyl fumarate. *J. Food Sci.*, **47**, 1710–1712.
2) Wang, H. H., Sun, D. W. and Kuang, R. (2001) Inhibition of *Escherichia coli* by dimethyl fumarate. *Int. J. Food Microbiol.*, **65**, 125–130.
3) Yazdi, R. M. and Mrowietz, U. (2008) Fumaric acid esters. *Clin. Dermatol.*, **26**, 522–526.
4) De Haan, P., von Blomberg-van der Flier, B. M. E., de Groot, J., Nieboer, C. and Bruynzeel, D. P. (1994) The risk of sensibilization and contact urticaria upon topical application of fumaric acid derivatives. *Dermatology*, **188**, 126–130.
5) Williams, J. D. L., Coulson, I. H., Susitaival, P. and Winhoven, S. M. (2008) An outbreak of furniture dermatitis in the U.K., *Br. J. Dermatol.*, **159**, 233–234.
6) Susitaival, P., Winhoven, S. M., Williams, J., Lammintausta, K., Hasan, T., Beck, M. H., Gruvberger, B., Zimerson, E. and Bruze, M. (2010) An outbreak of furniture related dermatitis ('sofa dermatitis') in Finland and the UK: history and clinical cases. *J. Eur. Acad. Dermatol. Venereol.*, **24**, 486–489.

7) Rantanen, T. (2008) The cause of Chinese sofa/chair dermatitis epidemic is likely to be contact allergy to dimethylfumarate, a novel potent contact sensitizer. *Br. J. Dermatol.*, **159**, 218–221.

8) Giménez-Arnau, A., Silvestre, J. F., Mercader, P., De la Cuadra, J., Ballester, I., Gallardo, F., Pujol, R. M., Zimerson, E. and Bruze, M. (2009) Shoe contact dermatitis from dimethyl fumarate: clinical manifestations, patch test results, chemical analysis, and source of exposure. *Contact Dermatitis*, **61**, 249–260.

9) Santiago, F., Andrade, P., Gonçalo, M., Mascarenhas, R. and Figueiredo, A. (2010) Allergic contact dermatitis to shoes induced by dimethylfumarate: A new allergen imported from China. *Dermatol. Online J.*, **16**(**3**), 3.

10) Foti, C., Zambonin, C. G., Cassano, N., Aresta, A., Damascelli, A., Ferrara, F. and Vena, G. A. (2009) Occupational allergic contact dermatitis associated with dimethyl fumarate in clothing, *Contact Dermatitis*, **61**, 122–124.

11) EU Directive 98/8/EC (1998) Directive 98/8/EC of the European parliament and of the council of 16 February 1998—concerning the placing of biocidal products on the market, European Commission, *Off. J. Eur. Communities*, L 123/1.

12) EU Directive 2009/251/EC (2009) Commission Decision of 17 March 2009 requiring Member States to ensure that products containing the biocide dimethylfumarate are not placed or made available on the market, European Commission, *Off. J. Eur. Communities*, L 74/32.

13) European Commission. Rapid Alert System for non-food consumer products (RAPEX), http://ec.europa.eu/consumers/dyna/rapex/rapex_archives_en.cfm (cited 18 August, 2010).

14) Doumit, J. and Pratt, M. (2009) Allergic contact dermatitis from dimethylfumarate after contact with Chinese sofa. *Proceedings of ACDS 20th Anniversary Meeting*, 12.

15) Hansson, C. and Thörneby-Andersson, K. (2003) Stereochemical considerations on concomitant allergic contact dermatitis to ester of the *cis-trans* isomeric compounds maleic acid and fumaric acid. *Skin Pharmacol. Appl. Skin Physiol.*, **16**, 117–122.

16) English, J. S. C., Lovell, C. R. and Rycroft, R. J. G. (1985) Contact dermatitis from dibutyl maleate. *Contact Dermatitis*, **13**, 337–338.

17) Lammintausta, K., Zimerson, E., Winhoven, S., Susitaival, P., Hasan, T., Gruvberger, B., Williams, J., Beck, M. and Bruze, M. (2010) Sensitization to dimethyl fumarate with multiple concurrent patch test reactions. *Contact Dermatitis*, **62**, 88–96.

18) Lamas, J. P., Sanchez-Prado, L., Garcia-Jares, C. and Llompart, M. (2009) Determination of dimethyl fumarate in desiccant and moldproof agents using ultrasound-assisted extraction gas chromatography with electron-capture detection. *J. Chromatogr. A*, **1216**, 5755–5758.

19) Lamas, J. P., Sanchez-Prado, L., Regueiro, J., Llompart, M. and Garcia-Jares, C. (2009) Determination of dimethyl fumarate and other potential allergens in desiccant and antimold sachets. *Anal. Bioanal. Chem.*, **394**, 2231–2239.

20) Narizzano, R., Risso, F., Venturelli, G., Devia, C., Carlini, E. and Maggiolo, S. (2009) Gas-chromatography-mass spectrometry analysis of dimethyl fumarate in consumer products. *J. Chromatogr. A*, **1216**, 6762–6766.

21) Liu, Y. F., Zhou, M. H., Li, D., Zhai, C. P., Li, Q. Z., Zheng, J. G., Yue, D. L. and Xiao, D. H. (2009) Determination of dimethyl fumarate in leather and leather products by GC/MS-SIM. *J. Chin. Mass Spectrom. Soc.*, **30** (extra issue), 222–224.

22) Japanese Industrial Standards Committee (2006) JIS K0123: General rules for gas chromatography /mass spectrometry.

23) Nakamura, M. (2008) Quality assurance and quality control of instrumental analysis. In *Analytical Methods for Pesticides and Other Organic Chemicals by GC/MS and LC/MS* (Kobayashi, H. and Nakamura, K., Eds.), Soft Science, Tokyo, pp. 66–83.

24) Hasan, T., Zimerson, E. and Bruze, M. (2010) Persistent shoe dermatitis caused by dimethyl fumarate. *Acta Derm. Venereol.*, **90**, 554–555.

25) Lammintausta, K., Zimerson, E., Hasan, T., Susitaival, P., Winhoven, S., Gruvberger, B., Beck, M., Williams, J. D. and Bruze, M. (2010) An epidemic of furniture-related dermatitis: searching for cause. *Br. J. Dermatol.*, **162**, 108–116.

TRANSFER OF PHTHALIC ACID DIESTERS FROM MODEL PVC SHEET TO SKIN SURFACE

Kawakami T[1], Isama K[1], Jinno H[1], Matsuoka A[2], Nishimura T[1]

[1]Division of Environmental Chemistry, [2]Division of Medical Devices, National Institute of Health sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo, Japan

Introduction

Chemicals used in household products may diffuse to indoor environments, and humans may inhale these chemicals. Some of these chemicals are adsorbed on house dust, and humans may ingest the house dust[1]. In addition, some chemicals may be transferred to the skin surface, and human exposure to these chemicals by skin absorption or hand-to-mouth contact has been indicated[2]. Thus, studies investigating the transfer of chemicals, for example, parabens[3], bisphenol-A[4] and fragrances[5], to human skin surfaces have been carried out. Phthalic acid diesters (PAEs) are widely used as plasticizers in various products, particularly those made from polyvinyl chloride (PVC). Since PAEs migrate easily from plastic products, and their content in plastic materials is high, these chemicals are of great concern globally. Although oral exposure of children to PAEs by chewing or licking toys and childcare products has been studied[6], the transfer of PAEs from household products to skin surfaces have not been investigated. Thus, we examined the transfer of PAEs from PVC household products to forearm skin surfaces using model PVC sheets. We have already surveyed the PAE contents of PVC household products in Japan[7], and reported that high detection frequencies and large amounts of di(2-ethylhexyl) phthalate (DEHP). In addition, we reported that diisononyl phthalate (DINP) and di-*n*-butyl phthalate (DBP) were also detected in PVC household products. Thus, we selected these PAEs as the target chemicals for this study. It was thought that sebum was an important factor in PAEs transfer to skin surface, and it has been reported that triglyceride (TG) is the dominant component in human sebum[8]. Therefore, we examined the relationships between the amounts of PAEs transferred to the skin surface and of TG collected from the skin surface.

Materials and methods

Eleven healthy adult subjects, seven men and four women, aged 31-57 years, participated in this study. The ethical review boards of the National Institute of Health Sciences approved this study (No.175). Written informed consent was obtained from all subjects.

Environmental analysis grade DBP, DEHP, and the deuterated PAEs [DBP, DEHP, di-*n*-octyl phthalate] used as internal standards were obtained from Kanto Chemical Co., Inc. DINP (CAS. 28553-12-0) was obtained from Wako Pure Chemical Ind., Ltd. Pesticide residue grade ethanol, acetone, and hexane were obtained from Kanto Chemical Co., Inc., Wako Pure Chemical Ind., Ltd. and Sigma-Aldrich, respectively.

PAEs analysis grade anhydrous sodium sulfate was obtained from Wako Pure Chemical Ind., Ltd. TG analysis was performed using a TG analytical kit purchased from Bio Vision. Triton-X 100 used for TG analysis was obtained from Sigma-Aldrich. Deionized water was produced by Milli-Q Synthesis A10 (Millipore). All utensils made of glass, metal, or Teflon were heated at 250°C for more than 12 h to prevent contamination. DEHP and DBP used for the production of model PVC sheets were obtained from Tokyo Chemical Ind., Co. DINP (Cas.28553-12-0) used for the PVC sheets was purchased from Wako Pure Chemical Ind., Ltd. The PVC polymer (S-1003) and stabilizer (AC-255) used for the PVC sheets were industrial materials. The concentrations of PAEs in the PVC sheet were determined by GC/MS[7] and these are listed in Table 1.

A square model PVC sheet (1.5 cm x 1.5 cm = 2.25 cm^2, weight and thickness approximately 83 mg and 0.3 mm, respectively) was used for this study. The PVC sheet was contacted with inside of the forearm, and fixed by a rectangular silicone mat and surgical tape. After 30 min, the sheet was removed and the part of the skin surface contacted with the sheet was wiped with a clean applicator wetted with ethanol. The applicator was then placed in a test tube and PAE was extracted with acetone. The sample solution was dehydrated with anhydrous sodium sulfate, and the volume was adjusted to 10 ml. Finally, an internal standard solution was added to 1 ml of the sample solution and the sample was analyzed by GC/MS. The transfer experiment for each dose level was carried out in triplicate (n=3). Control samples (using a Teflon sheet of the same size) and blank samples were also analyzed to measure the PAEs background levels and to confirm contamination during the experimental process. The PAE amounts transferred to the skin surface were calculated as the average of three sheets. These experiments were carried out twice per subject [1st: DBP 28%, DEHP 15%, 28%, 37%, DINP 28%; 2nd: DBP 15%, DINP 15%, two-mixed sheets (DEHP and DBP, DEHP and DINP)] on different days. A control sample was used to measure the TG amounts. The control sample solution (9 ml) was concentrated and dried under a gentle N_2 stream. The residue was dissolved in 5% Triton-X aqueous solution and the TG was analyzed using a TG measurement kit according to the Bio Vision protocol.

Table 1. Concentrations of PAEs in the model PVC sheet (n=3).

PAEs	Blending ratio of PAE (%)	Average (%)	SD	CV (%)
DBP	15	11.9	0.47	3.9
	28	24.5	0.58	2.4
	14[a]	11.4	0.18	1.6
DEHP	15	12.3	0.22	1.8
	28	23.5	0.14	0.6
	37	33.6	2.0	5.8
	14[a]	12.5	0.13	1.0
	14[b]	12.3	0.54	4.4
DINP	15	13.1	0.16	1.2
	28	26.1	0.72	2.8
	14[b]	13.4	0.24	1.8

[a]mixture of DEHP and DBP. [b]mixture of DEHP and DINP.

Results and discussion:

DEHP was detected in the control samples collected from all subjects (n=22, 0.013-0.11 μg/cm^2). DBP and DINP were not detected in the control samples collected from all subjects, excepting subject D. DBP was detected in the control samples collected from subject D (0.14 and 0.48 μg/cm^2). In a previous study, benzyl butyl phthalate and DBP were detected at levels of 0.79 and 0.90 ng/cm^2 (as median) in the wiped samples collected from children's skin surfaces[9]. The background amounts of DEHP detected on skin surfaces in this study were higher than those of other PAEs because DEHP is used with high frequency and in large amounts in PVC household products[7]. Stapleton et al. determined brominated flame retardants on skin surfaces (average 251 pg/cm^2)[2]. Methyl paraben has been detected on forefinger skin surfaces (approximately 0.2 μg/cm^2, maximum value)[3]. These differences in detection levels among chemicals used in household products probably reflect their physico-chemical properties and usage.

Noticeable differences in the amounts of PAEs transferred to the forearm skin surface were not observed among all subjects, except in the case of the 28% and 37% sheets for subjects D and I (Fig.1, for example DEHP). Subjects D and I were women, and they did not use cosmetic items before these experiments. The reason for this high transferability is still unknown. The amounts of DEHP transferred from the 28% and 37% sheets were relatively high compared to those transferred from the 15% sheet. However, differences between the 28% and 37% sheets were not observed (Fig. 1). The amounts of each PAE transferred from the mixed sheets were higher than those from single component sheet (15% sheet). The plasticity of the mixed sheet was higher than that of the 15% sheet because of the large total amount of plasticizer. Thus, the PAEs included in the mixed sheet bled easily on contact with the skin. To examine the transferability of PAEs from the PVC sheet to the skin surface, the transferred amounts of DBP or DINP were compared with that of DEHP for the mixed sheets (Fig. 2). The relationships between the transferred amounts of DBP or DINP and that of DEHP were observed; the slopes of their regression lines were 0.503 or 1.16, respectively. The ratios of DBP/DEHP and DINP/DEHP in the mixed sheets were 0.912 and 1.09 (w/w), respectively. Thus, the order of the transferability of these PAEs to the skin surface might be DINP, DEHP, DBP. In addition, the average amount of PAEs transferred to the skin surface from the 15% sheet was correlated with their octanol-water partition coefficient (K_{ow}). Thus, the hydrophobic properties of the PAEs may influence their transferability to the skin surface from PVC sheet.

The average amount of TG collected from the skin surfaces of all subjects was 9.3 nmol/cm^2 (n=22, 3.4-18.3 nmol/cm^2). This value was relatively high compared to those in a previous study that measured forearm sebum using a sebumeter[10], and slightly lower than in another previous study which used standardized adhesive patches designed to collect sebum[11]. Generally, men have higher amounts of sebum than woman[12]. However, differences in sebum amounts, based on age and sex, were not observed among

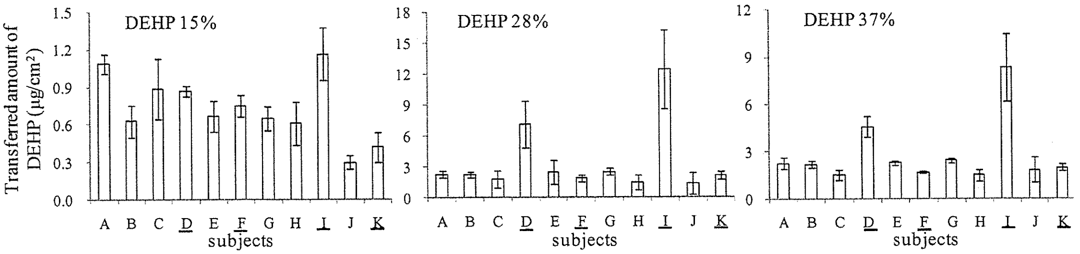


Fig. 1. Amounts of DEHP transferred from model PVC sheet to the skin surface (average ±SD).
(Alphabetical order means in order of age and underline means female subject)

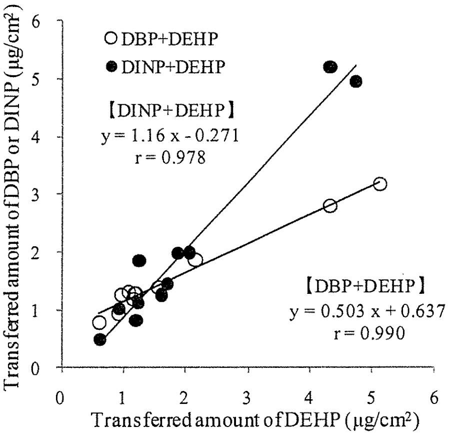


Fig. 2. Relationships between amount of DEHP and DBP or DINP transferred from mixture sheet.

the subjects in this study. No obvious relationship between the amounts of PAEs transferred to the skin surface and the amounts of TG collected from subject's skin was observed. It was thought that the PAEs were transferred to a very thin layer of skin surface, although the TG collected in this study might be collected not only from this very thin layer but also from under the thin layer of surface skin. Therefore, the method of sampling sebum on the skin surface might affect examination of the relationship between PAEs transferred and TG. In addition, TG is composed of several kinds of fatty acids and other compounds such as wax-esters and squalene, which are also components of sebum on the skin surface. Thus, it is necessary to examine the relationships between PAEs transferred to the skin surface and sebum components to evaluate the transferability of PAEs to the skin surface.

Acknowledgments:

The authors wish to thank Dr. Tatsuhiro Niino (Mitsubishi Chemical Medience) and Dr. Hiroaki Todo (Assistant Professor, Faculty of Pharmaceutical Sciences, Josai University) for their helpful advice during this study. This work was supported by grant H21-Kagaku-009 from the Ministry of Health, Labor, and Welfare of Japan.

References:

1. Kanazawa A, Kishi R. (2009); *Jpn J Hyg.* 64: 672-682
2. Stapleton MH, Kelly MS, Allen GJ, Mcclean DM, Webster FT. (2008); *Environ Sci Technol.* 42: 3329-3334
3. Shibata H, Tsuda T. (2002); *BUNSEKI KAGAKU.* 51: 397-401
4. Biedermann S, Tschudin P, Grob K. (2010); *Anal Bioanal Chem.* 398: 571-576
5. Api MA, Bredbenner A, McGowen M, Niemiera D, Parker L, Renskers K, Selim S, Sgaramella R, Signorelli R, Tedrow S, Troy W. (2007); *Regul Toxicol Pharmacol.* 48: 279-283
6. Sugita T, Kawamura Y, Tanimura M, Matsuda R, Niino T, Ishibashi T, Hirabayashi N, Matsuki Y, Yamada T, Maitani T. (2003); *J Food Hyg Soc Japan.* 44: 96-102
7. Kawakami T, Isama K, Matsuoka A.(2011); *J Environ Sci Health Part A.* in press
8. Greene SR, Downing TD, Pochi EP, Strauss SJ. (1970); *J Invest Dermatol.* 54: 240-247
9. U.S. EPA. *http://www.epa.gov/heasd/ctepp/* (accessed April 14, 2011)
10. Tanaka A, Seki T, Ochiai H, Tazawa K. (1999); *J Nurs Soc Toyama Med Pharma Univ*, 2: 49-58
11. Thiele JJ, Weber US, Packer L. (1999); *J Invest Dermatol.* 113: 1006-1010
12. Inomata N. (1975); *Rinsho Derma.* 29: 349-357

Osteoblast Compatibility of Calcium-Incorporated Ti-Zr-Nb Alloys

Isama K.,[1] Kawakami T.,[1] Tsuchiya T.,[2] and Matsuoka A.[3]

[1]Division of Environmental Chemistry, National Institute of Health Sciences, Tokyo 158-8501, Japan, [2]Medical Center for Translational Research, Osaka University Hospital, Osaka 565-0871, Japan, and [3]Division of Medical Devices, National Institute of Health Sciences, Tokyo 158-8501, Japan

Summary

Ca was incorporated into the surface of the Ti-Zr-Nb alloys in the $CaCl_2$ treatment and $Ca(OH)_2$ treatment following the NaOH treatment. According to the XRF analysis, the larger amount of Ca was incorporated in the $Ca(OH)_2$ treatment than in the $CaCl_2$ treatment. The Ca-incorporated Ti-Zr-Nb alloys were evaluated in the cytotoxicity and osteoblast compatibility *in vitro*. The untreated Ti-6Al-4V showed cytotoxicity, but Ti-Zr-Nb alloys did not show cytotoxicity with or without treatment. The Ca-incorporated Ti-Zr-Nb alloys showed good osteoblast compatibility. In Ti-Zr, Ti-Zr-4Nb, pure Ti and Ti-6Al-4V, the $Ca(OH)_2$ treatment enhanced the osteoblastic differentiation than the $CaCl_2$ treatment.

Introduction

Ti alloys are used as materials of bone fixations and artificial joints in orthopedics. Ti-6Al-4V is used generally, but it is known that vanadium ions have strong cytotoxicity. We reported Nb ions promoted the differentiation of osteoblasts[1-3] and Ti-Zr-Nb alloys had good mechanical property and osteocompatibility *in vitro* and *in vivo*.[4,5] On the other hand, the materials with a high apatite-forming ability in a simulated body fluid are expected to bond directly with the bone *in vivo*.[6] Ti alloys which have been alkali- and heat-treated to improve the apatite-forming ability have been applied clinically. Furthermore, the Ca incorporation into the alkali-treated Ti alloys have been attempted to achieve an even higher apatite-forming ability. We examined the Ca incorporation into the Ti-Zr-Nb alloys and confirmed Ca-incorporated Ti-Zr-Nb alloys had a high apatite-forming ability. In this study, we evaluated the cytotoxicity and osteoblast compatibility of the Ca-incorporated Ti-Zr-Nb alloys.

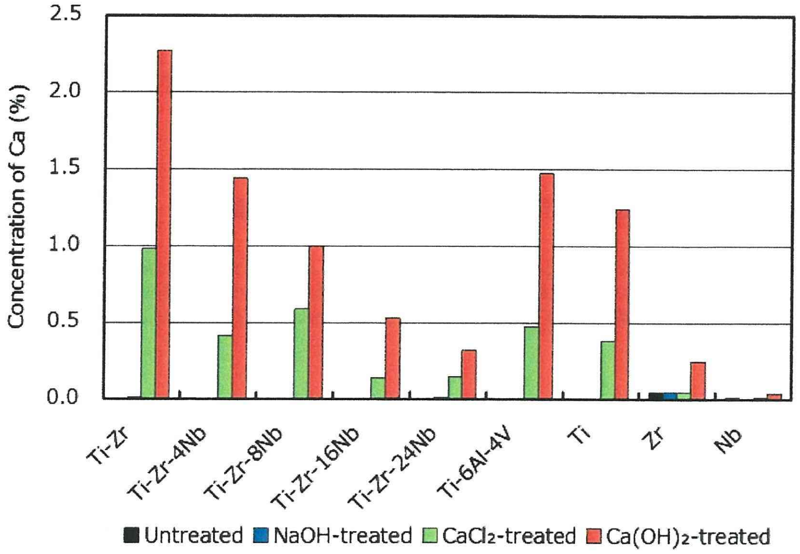


Fig. 1 Calcium amount of the surface of the samples by XRF using the fundamental parameter method.

Materials and Methods

Ti-Zr. Ti-Zr-4Nb. Ti-Zr-8Nb. Ti-Zr-16Nb and Ti-Zr-24Nb were used in this experiment. In addition. pure Ti. Zr and Nb were used as structural elements of the Ti-Zr-Nb alloys and Ti-6Al-4V was used as a comparison. All samples were 14.0 mm in diameter and 1.0 mm in thickness.

The samples were soaked in NaOH aqueous solution at 60°C for 24 hr (NaOH treatment). Subsequently. the NaOH-treated samples were soaked in $CaCl_2$ aqueous solution ($CaCl_2$ treatment) or $Ca(OH)_2$ aqueous solution ($Ca(OH)_2$ treatment) at 60°C for 24 hr. The Ca amount of the $CaCl_2$-treated and $Ca(OH)_2$-treated surfaces was measured by X-ray fluorescence spectrometry (XRF) using the fundamental parameter method.

The cytotoxicity of the samples was evaluated by the colony formation assay with direct contact using Chinese hamster lung fibroblast V79 cells.[7] The osteoblast compatibility of the samples was evaluated with normal human osteoblasts (Cambrex Bio Science Walkersville. Inc.) cultured in α-modified minimum essential medium supplemented with 10% fetal bovine serum and 5 mM disodium β-glycerophosphate. The osteoblast proliferation was estimated with the cell number measured by WST-8 assay.[7] The osteoblastic differentiation was estimated with the alkaline phosphatase (ALP) activity measured using p-nitrophenylphosphate as a substrate.[7]

Results and Discussion

All Ti alloys and pure Ti incorporated almost double amount of Ca in the $Ca(OH)_2$ treatment compared with the $CaCl_2$ treatment. Particularly. Ti-Zr. Ti-Zr-4Nb. pure Ti

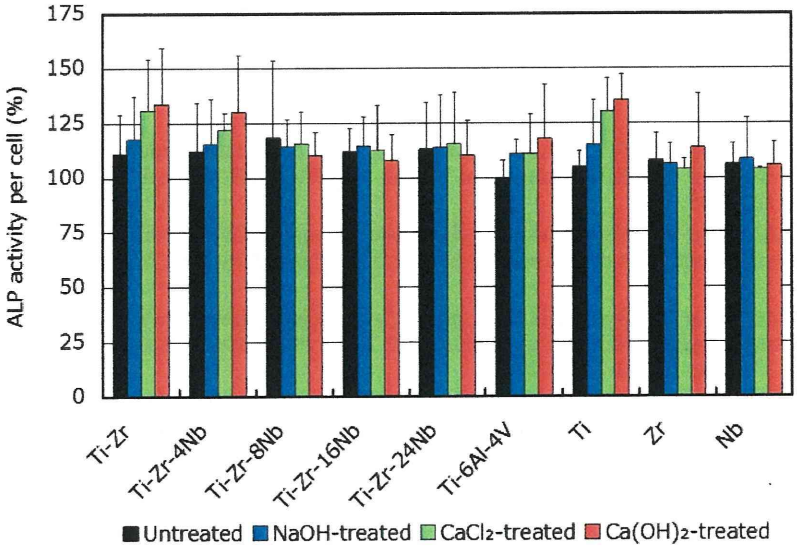


Fig. 2 Osteoblastic differentiation on the samples estimated with the ALP activity per cell measured using p-nitrophenylphosphate as a substrate.

and Ti-6Al-4V incorporated large amount of Ca. Pure Zr incorporated Ca only in the Ca(OH)$_2$ treatment, and pure Nb did not incorporate Ca in both treatments (Fig. 1).

The untreated Ti-6Al-4V showed cytotoxicity, but treated Ti-6Al-4V showed less cytotoxicity. Other samples did not show cytotoxicity with or without treatment. Compared with on the untreated Ti-6Al-4V, the cell number of osteoblasts increased on other samples. This result was supported by the cytotoxicity of the samples (data not shown).

The ALP activity of osteoblasts cultured on all other samples increased compared with on the untreated Ti-6Al-4V. Additionally, in Ti-Zr, Ti-Zr-4Nb, pure Ti and Ti-6Al-4V, the ALP activity of osteoblasts was promoted in order of by the NaOH, CaCl$_2$ and Ca(OH)$_2$ treatment (Fig. 2). In the previous study, the apatite-forming ability of Ti-Zr, Ti-Zr-4Nb, pure Ti and Ti-6Al-4V increased in order of by the NaOH, CaCl$_2$ and Ca(OH)$_2$ treatment. These results implied the osteoblastic differentiation was enhanced on the materials with a high apatite-forming ability.[8]

Conclusions

The Ca-incorporated Ti-Zr-Nb alloys showed good osteoblast compatibility. In Ti-Zr, Ti-Zr-4Nb, pure Ti and Ti-6Al-4V, the Ca(OH)$_2$ treatment enhanced the osteoblastic differentiation than the CaCl$_2$ treatment. The large amount of Ca incorporation would induce good osteoblastic differentiation cultured on the materials.