Table 2. Recovery tests of organophosphorus pesticides from fruits and vegetables by acetonitrile extraction

Pesticide tested	fortified (μg/g)	Average recovery, % (RSD)			
		apple extract	cucumber extract	tomato extract	strawberry extract
Chlorpyrifos	0.01	85.7 (4.48)	85.7 (4.09)	82.1 (1.3)	94.5 (7.3)
Diazinon	0.01	85.1(2.64)	83.5 (6.72)	91.3 (3.84)	95.5 (3.9)
Fenitrothion	0.01	98.8 (15.3)	88.4 (6.79)	89.9 (1.47)	89.3 (7.1)
Isoxathion	0.01	102 (22.9)	92.5 (22)	84.3 (5.42)	99.5 (12.2)
Benthiocarb	0.1	87.1 (3.58)	89.2 (4.02)	88.5 (5.05)	92.6 (6.9)
Carbaryl	0.1	90.5 (6.75)	88.2 (5.9)	89.2 (7.51)	95.3 (10.2)
Fenocarb	0.1	91.8 (3.5)	90.5 (8.01)	84.8 (4.05)	98.5 (9.8)
Isoprocarb	0.1	86.2(10.2)	85.1 (3.09)	89.4 (5.02)	90.8 (6.9)

RSD = Relative standard deviation.

Table 3. Chlorine consumption and residual chlorine within fruit and vegetable extracts (acetonitrile) at 25°C for 15 min

Chlorine dose to the extract (mg/l)	Chlorine-consumed and remained in the extracts (as HOCl, mg/l)							
	Apple		Cucumber		Tomato		Strawberry	
	consumed	remained	consumed	remained	consumed	remained	consumed	remained
5	3.4	0	5	0	5	0	5	0
12.5	10.5	1.5	12.5	0	12.5	0	10	0
25	21.2	3.8	24.5	0.5	23.4	1.6	25	0
50	46.7	3.3	47.6	2.4	46.5	3.5	50	0
100	93	7	91.4	8.6	90	10	100	0
200	183.1	16.9	177	23	179.5	20.5	200	0
350	321.1	28.9	315	35	315	35	349	1
500	456.9	43.1	439	61	443	57	495	5
750	679.7	70.3	664	86	651	99	731	19
1000	900	100	890	110	885	115	963	37
2000	1831	169	1770	230	1795	205	1940	60

Chlorine consumptions of acetonitrile extract containing different matrices and ChE inhibition assays

We have confirmed recovery rates for pesticides from fruit and vegetable samples by acetonitrile extraction and clean-up procedure (Table 2). It is also important to discern how the distribution of oxidation products varies with experimental conditions, such as hypochlorite oxidation and ChE-inhibiting assays. Our previous papers (Onodera *et al.*, 1992; Kanno *et al.*, 2012) revealed that excess hypochlorite in water at pH 7.0 oxidised the P=S pesticides stoichiometrically within 5 min into their P=O analogues. The resulting products were comparatively stable in water at low concentrations of active chlorine (less than 100 mg/l) in an acidic solution of pH 6.0. In addition, greater ChE-inhibiting activities (I_{50} = 0.004-0.1 μM) were also found for the oxidative compounds formed by the hypochlorite oxidations of diazinon, ethion, isoxathion, parathion, cumafos, phorate, azinfos-methyl, and runnel in water.

Table 3 summarises the chlorine consumptions by the acetonitrile extracts containing fruit and vegetable matrices and the residual chlorine concentrations in these extracts after exposure to various chlorine doses at 25°C for 15 min. As shown in the table, the chlorine consumptions depended strongly on the species of fruit or vege-

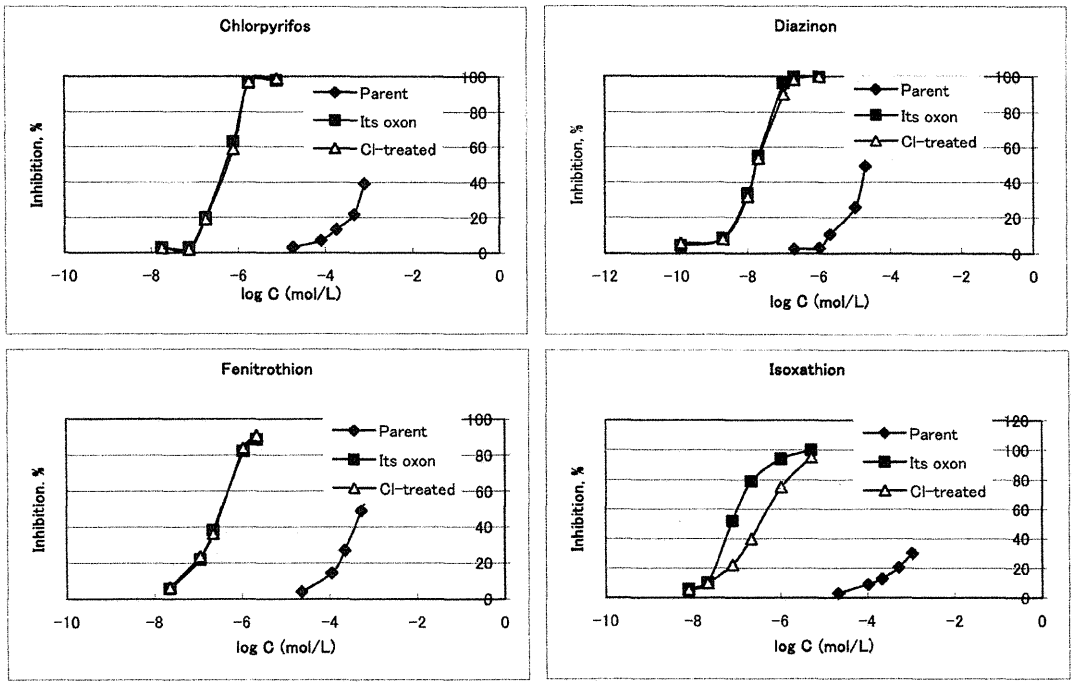

Fig. 2. Plot of ChE-inhibition activity vs. concentration of organophosphorus pesticides before and after hypochlorite oxidation in phosphate buffer solution. Each pesticide solution (pH 7.0) was treated with excess hypochlorite (final concentration, 100 mg/l) at 25°C for 15 min. The mixture of the enzyme solution and pesticide solution was incubated at 37°C for 30 min, after which the anti-ChE activity was assessed. The mean of the inhibition (%) obtained in three test runs was plotted.

table examined and the concentrations of active chlorine exposed. At the chlorine dose of 100 mg/l (in the form of HOCl), extracts of apple-, cucumber-, tomato-, and strawberry consumed 93, 91.4, 90, and 100% of the total amount of chlorine added, respectively, and they showed a very low concentration of residual chlorine (less than 10 mg/l). The largest consumption of active chlorine and lowest remaining residual chlorine were observed for strawberry extract, even at a high chlorine dose of 500 mg/l. These chlorine consumptions in Table 3 reflect the reductive powers of fruit and vegetable extracts containing several reducing reagents, such as sugars, hydrocarbons, metal ions and Vitamin C. It is known that the commercial strawberry, tomato, cucumber, and apple contain 80, 20, 13, and 3 mg/100 g of vitamin C, respectively (Standard Tables of FOOD Composition in Japan, 2010). On the other hand, Kim *et al.* (2000) reported that treatment of nine organophosphorus pesticides with excess bromine in organic solvent (pure acetonitrile) is a rapid and efficient method of achieving their oxidation prior to the ChE-inhibition assays. However, the previous study lacked information on the oxidation conditions in the presence of different matrices in fruit and vegetable extracts and the chemical fate of oxons in non-aqueous solutions.

Table 4 presents the inhibition 50% (I_{50}) and 20% (I_{20}) values towards ChE of the horse serum observed for chlorpyrifos, diazinon, fenitrothion, and isoxathion in fruit and vegetable extracts before and after solid phase clean-up using ENV™-Carb column and following oxidation with hypochlorite with a chlorine dose of 1,000 mg/l (as active Cl) and at 25°C for 15 min. The I_{50} and I_{20} values of individual chlorinated acetonitrile extracts calculated from these ChE-inhibiting curves were in agreement with those observed for aqueous pesticide solutions after treatment with hypochlorite, as shown in Fig. 2. The GC-MS determinations also indicated that exposure to high chlorine doses oxidised the P=S pesticides within a short time into their P=O analogues (excepted for the isozathion products), and the resulting products were comparative-

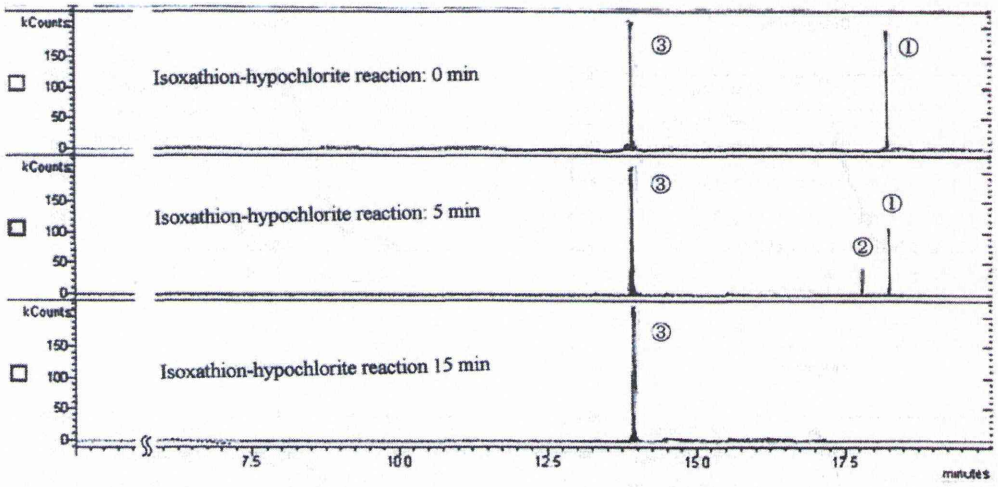

Fig. 3. Example of gas chromatograms (total ion currents) of the ethyl acetate extracts from the isoxathion-hypochlorite reactions in phosphate buffer solution of pH 7.0 and hypochlorite concentration of 100 mg/l. ① = isoxathion; ② = isoxathion-oxon; and ③ = phenathrene-d10 as surrogate compound.

Table 4. The inhibition 50% (I_{50}) and 20% (I_{20}) values of organophosphorus pesticides in acetonitrile extracts of fruit and vegetable samples, before and after clean-upped by the Standard method in Japan and folowing the hypochlorite-oxidation.

Pesticides tested (fortified)	I_{50}/I_{20} values (ug/g)*1 of chlorinated acetonitrile extracts							
	Apple		Cucumber		Tomato		Strawverry	
	original	clean-upped	original	clean-upped	original	clean-upped	original	clean-upped
Chlorpyrifos (0.2 μg/g)	0.14/0.04	0.15/0.04	0.15/0.04	0.14/0.04	0.13/0.04	0.14/0.04	0.13/0.04	0.13/0.04
Diazinon (0.1 μg/g)	0.005/0.001	0.004/0.001	0.004/0.001	0.005/0.001	0.004/0.001	0.005/0.001	0.006/0.001	0.005/0.001
Fenitrothion (0.2 μg/g)	0.13/0.04	0.13/0.004	0.14/0.04	0.14/0.04	0.14/0.04	0.15/0.04	0.16/0.04	0.16/0.04
Isoxathion (0.2 μg/g)	0.03/0.01	0.03/0.01	0.03/0.01	0.04/0.01	0.03/0.01	0.03/0.01	0.03/0.01	0.03/0.01

*1 These values indicate the average of three test runs, calculated from each of their ChE-inhibition curves.

ly stable in the chlorinated fruit and vegetable extracts. Therefore, the different matrices in the fruit and vegetable extracts did not interfere with the hypochlorite oxidations of organophosphates and their ChE-inhibiting assays.

ChE inhibition assays for organophosphorus pesticide combinations

A number of organophosphate mixtures, and pesticide and carbamate mixtures such as a combination of isoxathion-DDVP, acephate-NAC, and so on are often utilised to control insects and pests worldwide. Where a mixture of pesticides is present, the inhibition of AChE represents the total cholinesterase activity. However, it is not clear whether a mixture of pesticides causes a greater inhibition effect than the sum of its individual effects, (i.e., a synergistic effect). Fig. 4 shows the influence of DDVP as

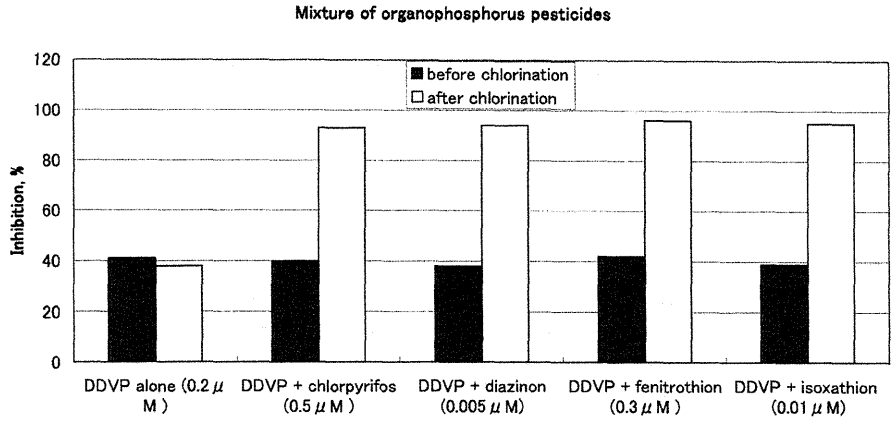

Fig. 4. Influence of coexisting DDVP on the formation of organophosphorus compounds (P=O analogues) during organothiophosphorus (P=S) pesticides–hypochlorite reactions. Each P=S pesticide and DDVP mixture, having the ability to inhibit 40% of horse serum cholinesterase before chlorination, was treated with hypochlorite (100 mg active Cl/l) for 15 min at 25°C for subsequent ChE assay.

a typical P=O pesticide on the P=S compound-hypochlorite reactions and following the ChE inhibition assay. A certain amount of each P=S compound exhibiting I_{50} in the ChE activity, was examined using the DDVP solution (0.2 μM). The presence of DDVP did not interfere with P=O compound formation in either the hypochlorite oxidation or the subsequent enzymatic assays. In addition, the inhibitory effects of a combination of DDVP and several P=S pesticides were found to be additive, although some pesticides are known to be potentiators or antagonists of the toxicity of other organophosphates *in vitro* (Elhalwagy and Zaki, 2009). Previously, Richardson *et al.* (2001) and Tahara *et al.* (2005) evaluated the *in vitro* interaction between two or three combinations of organophosphates on ChE activity. When two or three mixtures were added simultaneously to brain tissue *in vitro*, they had an additive effect.

On the basis of the above-mentioned fundamental studies, we calculated the degree of enhancement of anti-ChE activities (I_{50}) and detection limits (I_{20}) for each solution of chlorinated pesticides (1,000 mg/l of active chlorine at 25°C for 15 min, followed by the incubation with ChE of horse serum at 37°C for 30 min). Higher anti-ChE activity (I_{50} = 0.006 and 0.03 μg/g) was found for the P=S compounds, such as diazinon and isoxathion, when these pesticides were treated with hypochlorite in fruit and vegetable extracts. Moderate anti-ChE activity (I_{50} = 0.15 and 0.13 μg/g) was also observed for the P=S compounds, such as chloropyrifos and fenitrothion, after chlorination. These I_{50} values and the detection limits (I_{20}) presented in Table 4 are not able to achieve the sensitivity of traditional chromatographic methods (detection limits: lower than 0.001 μg/g) . However, the combination method of ChE assay and hypochlorite oxidation in acetonitrile extracts may be useful as a screening tool which could allow the screening of hundreds of samples in a short period of time and a lower cost. In addition, anti-ChE activity can serve as a "toxicological index", a measure of the toxicity of sample.

Application to real fruit and vegetable samples

The combination method of ChE assay and hypochlorite oxidation was used to monitor the inhibitors present in environmental samples, according to previous papers (Onodera *et al.*, 1992; Kawakami *et al.*, 2008; Kanno *et al.*, 2012). The organic concentrates obtained from fruit and vegetable samples that had been spiked with organophosphorus pesticides, followed by hypochlorite oxidation (1,000 mg active Cl/l), were tested at three doses for their ChE-inhibiting activity. Dose–response relationships between sample volume and ChE-inhibiting activity were clearly apparent (Table 5). Substantial ChE-inhibiting activity was observed in the chlorinated fruit and vegetable extracts, compared with the lower level of activity observed in the non-chlorinated concentrates. Thus, the ChE inhibitors were definitely recovered from the sam-

Table 5. Determination of organophosphorus pesticide in fruirt and vegetable samples before and after spiked with pesticide by ChE-inhibition assays, after extrsction with acetoritrile and hypochlorite oxidation.

Pesticide tested	fortified		Apple		Cucumber		Tomato		Strawberry	
	ng/g		inhibition %	S.D.	inhibition %	S.D.	inhibition %	S.D.	inhibition %	S.D.
Diazinon	0	(original)	ND*		ND		ND		ND	
	3.13		25.4	5.02	28.8	7.1	25.8	2.01	23.8	11.1
	6.25		46.2	8.57	45.1	11.6	43.5	7.15	44.3	5.8
	31.3		85.4	8.97	76.2	10.6	82.5	3.64	79.9	12.6
Fenitrotion	0	(original)	ND		ND		ND		ND	
	31.2		11.8	3.3	6.44	2.58	2.1	1.8	5.5	5.8
	62.5		23.2	4.16	11.5	9.05	18.5	1.58	15.3	3.55
	313		71.6	5.45	49.2	1.59	62.4	3.32	62.1	8.09
Chloropyrifos	0	(original)	ND		ND		ND		ND	
	156		34.8	6.38	47.5	18.6	45.8	29.7	33.6	10.8
	313		59.8	7.67	106	12.3	53.5	34.2	60.1	5.9
	625		89.4	8.12	115	13.5	85	7.16	85.8	9.25
Isoxathion	0	(original)	ND		ND		ND		ND	
	6.25		16.7	2.29	13.3	16.2	24.2	12	18.9	14.5
	31.3		53.6	6.33	53.6	9.75	51.6	6.98	49.9	7.4
	62.5		88.3	9.25	84.8	5.9	63.9	23.5	86.8	11.8

* ND = Not detected.

ples by acetritrile extraction and underwent the transformation of P=S pesticides into P=O compounds by hypochlorite oxidation. Several insecticides such as chlorpyrifos, DDVP, diazinon, fenitrotion, and isoxathion were not detected at all or were detected in trace amounts in the raw fruits and vegetables collected from the supermarket.

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—Regular Article—

有害物質含有家庭用品規制法で規制されている繊維製品中のトリス（2,3-ジブロムプロピル）ホスフェイト分析法の改定に向けた検討

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Study for the Revision of Analytical Method for Tris (2,3-dibromopropyl) phosphate with Restriction in Textiles

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The official analytical method for tris (2,3-dibromopropyl) phosphate (TDBPP), which is banned from use in textile products by the "Act on Control of Household Products Containing Harmful Substances", requires revision. This study examined an analytical method for TDBPP by GC/MS using a capillary column. Thermal decomposition of TDBPP was observed by GC/MS measurement using capillary column, unlike in the case of gas chromatography/flame photometric detector (GC/FPD) measurement based on a direct injection method using a capillary megabore column. A quadratic curve, $Y=2572X^{1.416}$, was obtained for the calibration curve of GC/FPD in the concentration range 2.0–100 μg/mL. The detection limit was 1.0 μg/mL under S/N=3. The reproducibility for repetitive injections was satisfactory. A pretreatment method was established using methanol extraction, followed by liquid-liquid partition and purification with a florisil cartridge column. The recovery rate of this method was ~100%. TDBPP was not detected in any of the five commercial products that this study analyzed. To understand the cause of TDBPP decomposition during GC/MS (electron ionization; EI) measurement using capillary column, GC/MS (chemical ionization; CI), GC/FPD, and gas chromatography/flame ionization detector (GC/FID) measurements were conducted. It was suggested that TDBPP might thermally decompose both during GC injection, especially through a splitless injection method, and in the column or ion sources. To attempt GC/MS measurement, an injection part comprising quartz liner was used and the column length was halved (15 m); thus, only one peak could be obtained.

Key words——tris (2,3-dibromopropyl) phosphate; organophosphate flame retardant; GC/MS; gas chromatography/flame photometric detector; textile; household product

緒　　言

トリス（2,3-ジブロムプロピル）ホスフェイト（TDBPP）は，セルローズ（繊維素），トリアセテート及びポリエステル生地の難燃剤として使用されてきたが,[1] ラット及びマウスによる動物実験で発がん作用を示すことが明らかになった.[2-4] そこで，TDBPPは，欧州の数ヵ国，米国，日本などにおいて使用禁止となった．さらに，International Agency for Research on Cancer（IARC，国際がん研究機関）は，1987年にTDBPPを発がん物質分類のグループ2A（ヒトに対しておそらく発がん性を示す：probably carcinogenic to humans）に分類した.[5]

日本では，1978年に「有害物質を含有する家庭用品の規制に関する法律」（家庭用品法）により，繊維製品にTDBPPを使用することが禁止され，公定法も定められた．しかし，その分析法は，日本薬局方原案作成要領[6]で原則使用しないこととされている有害性のあるベンゼンを用いた方法で精製し，分離能の低いパックドカラムを用いて，リン化合物のみに選択性がある炎光光度型検出器（リン用干渉フィルター）（flame photometric detector; FPD）付きガスクロマトグラフ（GC/FPD）で測定する方

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法である.[7] 家庭用品法では，公定法の結果に基づいて製品回収などの行政措置を行うことが原則となっている.「不適」事例報告があった1980年代前半，公定法で検査を実施し，TDBPPとほぼ同じ保持時間を持つ物質が検出されたためGC/MSを用いて確認同定したところ，TDBPPとは異なる物質であることが判明し，さらに，その物質以外に疑似物質が存在することも報告されている.[8] TDBPPの検査は，都道府県や政令市で，現在も毎年継続して行われている.[9] 近年,「不適」事例報告はないものの，防炎加工の必要性が高まり，新たなリン系難燃剤が使用される可能性があるため，より選択性の高い分析法の開発が求められている．そこで，ベンゼン等有害な試薬を使用しない精製法や，より選択性及び精度の高いキャピラリーカラムを使用したGC/MSを導入するための分析法を検討することとした．その分析法の検討過程で，TDBPP標準品が測定時に分解する現象が観察された．そこで，分解の影響が少ない直接注入のメガボアカラムを用いたGC/FPDで分析法を検討し，良好な回収率を得る前処理法を確立した．さらに，キャピラリーカラムを用いたGC測定時にTDBPPが分解する現象について原因究明を行い，解決策を検討したので報告する．

方　　法

1. 試料　市販の防炎加工繊維製品5製品（5部位）を試験試料とした.

2. 試薬　トリス（2,3-ジブロムプロピル）ホスフェイト（TDBPP）の標準試薬は複数のメーカーから購入した．1つは，和光純薬工業製の家庭用品試験用標準試薬（MW：697.61，含量90%以上）を用いた．そのほかに，Sigma-Aldrichから購入したものを用いたが，そのうち，SUPELCO製(MW：697.67，純度95.5%）についてLot番号の異なる2種類（A：Lot LB75285及びB：Lot LB83032V）を購入し，さらにFluka製（MW：697.61，純度98.3%）も入手した.

メタノールは和光純薬工業製LC/MS用，酢酸エチル，シクロヘキサン，*n*-ヘキサン及びアセトンは和光純薬工業製残留農薬分析用，*n*-ノナンは和光純薬工業製特級を用いた．フロリジルカートリッジカラムはWaters製のSep-Pack® plus Florisil® (910 mg/1.4 mL）を用いた.

3. 装置及び測定条件

3-1. 炎光光度型検出器（リン用干渉フィルター）付きガスクロマトグラフ（GC/FPD）（メガボアカラム測定）　GC装置は，Hewlett Packard製5890 Series II GCにFPD検出器を装着した装置を用いた．メガボアカラムは，DB-1（0.53 mmϕ×15 m，膜厚1.5 μm，J&W Scientific製）を用いた．キャリアーガス流量は，He 14.5 mL/minに設定した．カラム温度は，100℃（2 min）-20℃/min-290℃（7.5 min）にプログラミングし，注入口及び検出器温度は290℃に設定した．注入方法は直接注入法，注入量は1 μLとした.

3-2. GC/FPD（キャピラリーカラム測定）　GC装置はメガボアカラム測定と同じ装置を用いた．キャピラリーカラムは，DB-5（0.25 mmϕ×30 m，膜厚0.25 μm，J&W Scientific製）を用いた．キャリアーガス流量は，He 1 mL/minに設定した．カラム温度は，100℃（2 min）-20℃/min-290℃（10 min）にプログラミングし，注入口及び検出器温度は290℃に設定した．注入方法はスプリットレスで，注入量は1 μLとした.

3-3. GC/MS（electron ionization; EI測定）

3-3-1. 測定条件(1)　GC/MS装置は，Hewlett Packard製5890 Series II GCにMSD検出器（HP-5971）を装着した装置を用い，自動注入装置は，Hewlett Packard製7673型を用いた．キャピラリーカラムは，Inert Cap 5 MS/NP（0.25 mmϕ×30 m，膜厚0.25 μm，GLサイエンス製）を用いた．キャリアーガス流量は，He 1 mL/minに設定した．カラム温度は，100℃（2 min）-20℃/min-290℃（6 min）にプログラミングし，注入口及びインターフェイス温度は290℃に設定した．注入方法はスプリットレスで，注入量は1 μLとした．イオン化法はEI法で，イオン化電圧は70 eVとした．スキャンモード（SCAN: m/z＝50-550）及びselected ion monitoring（SIM）モードで測定した.

3-3-2. 測定条件(2)　GC/MS装置は，Hewlett Packard製6890 N GCに，日本電子製MSD検出器（JEOL JMS-Q1000GCK9）を装着した装置を用いた．キャピラリーカラムは，HP-5（0.25 mmϕ×15 m，膜厚0.25 μm, J&W Scientific製）を用いた．キャリアーガス流量は，He 2 mL/minに設定した．カラム温度は，100℃（2 min）-20℃/min-290℃

(6 min) にプログラミングし，注入口温度は 290℃に，インターフェイス温度は 250℃に設定した．注入方法はスプリットレスで，注入量は 1 μL とした．イオン化法は EI 法で，イオン化電圧は 70 eV，イオン源温度は 160℃とした．スキャンモード（SCAN: m/z=50–750）及び SIM モードで測定した．

3-4. GC/MS（chemical ionization; CI 測定）

3-4-1. 測定条件(1) GC/MS 装置は，Hewlett Packard 製 6890GC に MSD 検出器（HP-5973）を装着した装置を用いた．キャピラリーカラムは，HP-5MSI（0.25 mmφ×30 m，膜厚 0.25 μm，J&W Scientific 製）を用いた．キャリアーガス流量は，He 1 mL/min に設定した．カラム温度は，100℃ (2 min)–20℃/min–290℃ (6 min) にプログラミングし，注入口は 290℃，インターフェイス温度は 250℃，イオン源は 160℃に設定した．注入方法はスプリットレスで，注入量は 1 μL とした．イオン化法は CI 法で，反応ガスはメタンを用いた．m/z =50–750 の範囲でスキャン測定した．

3-4-2. 測定条件(2) GC/MS 装置は，Hewlett Packard 製 6890N GC に，日本電子製 MSD 検出器（JEOL JMS-Q1000GCK9）を装着した装置を用いた．キャピラリーカラムは，DB-5MS（0.25 mmφ ×30 m，膜厚 0.25 μm，J&W Scientific 製）を用いた．キャリアーガス流量は，He 1 mL/min に設定した．カラム温度は，100℃ (2 min)–20℃/min–290 ℃ (6 min) にプログラミングし，注入口は 290℃，インターフェイス温度は 250℃，イオン源は 160℃に設定した．注入方法はスプリットレスで，注入量は 1 μL とした．イオン化法は CI 法で，反応ガスはイソブタンを用いた．m/z=50–1000 の範囲でスキャン測定した．

3-5. 水素炎イオン化検出器付きガスクロマトグラフ（GC-FID） GC 装置は，Hewlett Packard 製 5890 Series II GC に flame ionization detector (FID) 検出器を装着した装置を用いた．キャピラリーカラムは，DB-5（0.25 mmφ×30 m，膜厚 0.25 μm，J&W Scientific 製）を用いた．キャリアーガス流量は，He 1 mL/min に設定した．カラム温度は，100℃ (2 min)–20℃/min–290℃ (10 min) にプログラミングし，注入口及び検出器温度は 290℃に設定した．注入方法はスプリットレスで，注入量は 1 μL とした．

3-6. ESI/MS 高分解能 MS 装置は，フーリエ変換-リニアイオントラップ型質量分析計 LTQ Orbitrap XL（Thermo Fisher Scientific 製）を用いた．試料はスプレーチップを用いて infusion 法で導入し，イオン化法には electron spray ionization (ESI) 法を用いた．試料溶液は，Positive モードでは MeOH/0.2％ギ酸水溶液（1：1）を，Negative モードでは MeOH/水（1：1）を用いて調製し，流速は 0.2 μL/min に設定した．スプレー電圧は 1.8 kV に，キャピラリー温度は 200℃とした．キャピラリー電圧は Positive mode は 35 V，Negative mode は－49.0 V とし，分解能は 10 万に設定した．ポリチロシン三量体由来のイオンをロックマス用とした（Positive mode: m/z=508.20783，Negative mode: m/z=506.19327）．精密質量の理論値及び測定値との質量差の計算には，Thermofisher Scientific の Xcalibur ver. 2.1/Qual Browser ソフトを使用した．

4. 試験溶液の調製 細切した試料 1 g をナス型フラスコに秤量し，メタノール溶液 50 mL を加え，30 分間 70℃で還流抽出した．抽出液をガラスろ過器でろ過し，200 mL のナス型フラスコに採取した．20 mL のメタノールで抽出に用いたガラス器具及び試料を洗浄して，洗液とろ液をあわせた．抽出液を 10 mL に濃縮し，50 mL の遠沈管に移した．精製水 10 mL，シクロヘキサン 10 mL を加え 3 分間激しく振とうした後，3000 rpm で 10 分間遠心分離を行い，シクロヘキサン層を分取した．さらに，シクロヘキサン 10 mL を加えて振とう後，シクロヘキサン層をあわせる操作を 2 回行った．抽出液を無水硫酸ナトリウムで脱水した．ろ液をロータリーエバポレーターで 2 mL に濃縮し，あらかじめヘキサン 10 mL で調製したフロリジルカートリッジカラムに負荷した．カラムをヘキサン 20 mL で洗浄した後，30％エタノール含有ヘキサン 30 mL で TDBPP を溶出させた．溶出液をナス型フラスコに採り，ロータリーエバポレーターで減圧濃縮し，アルゴン気流下で溶媒を留去した．残渣をヘキサン 1 mL に溶解し，GC/MS, GC/FPD 及び GC/FID 測定試料とした．

5. 検量線の作成 TDBPP 標準品（SUPELCO 製 B）1000 mg をアセトン 100 mL で溶解し，標準

原液（10000 μg/mL）を調製した．その溶液をヘキサンで希釈し，0.5, 1.0, 2.0, 10, 20, 40, 60, 80, 100 μg/mL となるように検量線用標準溶液を調製し，メガボアカラムを用いた GC/FPD 測定による検量線を作成した．

結果及び考察

1. 検出法の検討　詳細は後述するが，GC 注入口などでの TDBPP（Fig. 1）の分解現象が観察され，GC/MS による定量は困難なことが分かった．そこで，熱分解の影響が少ないメガボアカラムを用いた直接注入法で GC/FPD 測定を実施したところ，TDBPP の分解物はほとんど検出されなかった（Fig. 2）．今までに報告されているメガボアカラムを用いた測定[8,10,11]でも，分解現象について報告されておらず，現公定法で指定されているパックドカラムでも分解現象は観察されなかったと推測される．そこで，公定法に近いメガボアカラム（DB-1）を用いた直接注入 GC/FPD で定量法を検討した．

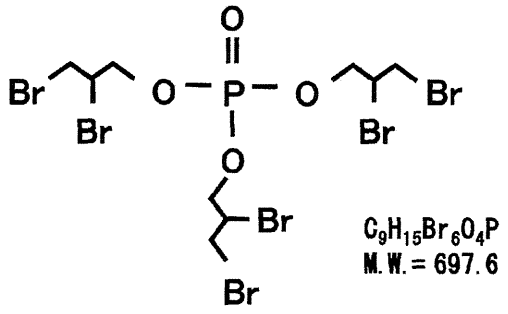

Fig. 1. Chemical Structure of TDBPP

2. 検量線（メガボアカラムによる GC/FPD 測定）　GC/FPD 測定による検量線は，2.0–100 μg/mL の範囲で，$Y=2572X^{1.416}$ の 2 次曲線を示す検量線が得られた．検出限界は，S/N=3 として 1.0 μg/mL であった．繰り返し注入の再現性も良好であった．

3. 液–液分配による極性物質の除去　繊維製品からの抽出法は，現公定法に従ってメタノールによる還流抽出法で行うこととした．まず，液–液分配法で，メタノール抽出液から極性物質を除去する方法を検討した．50, 250 μg の TDBPP をメタノール溶液（抽出液）2.5 mL とし，そこに水 2.5 mL 及び有機溶媒 2.5 mL（ヘキサン，シクロヘキサン又は酢酸エチル–ヘキサン混液）を加えて，TDBPP を有機溶媒層に再抽出可能かを検討した．TDBPP は，ヘキサン層に 48–55%，シクロヘキサン層に 62–79%，酢酸エチル–ヘキサン混液（2：3）層に 69–82%，酢酸エチル–ヘキサン混液（1：4）層に 63–84%が移行した．後述するが，酢酸エチル–ヘキサン混液では，フロリジルカラム操作時に回収率の低下が観察されたため，シクロヘキサンを用いることとした．すなわち，メタノール抽出液に同量の水とシクロヘキサンを加え，TDBPP をシクロヘキサン層に再抽出し，共存極性物質を除去した．

4. フロリジルカラムによる無極性（脂溶性）物

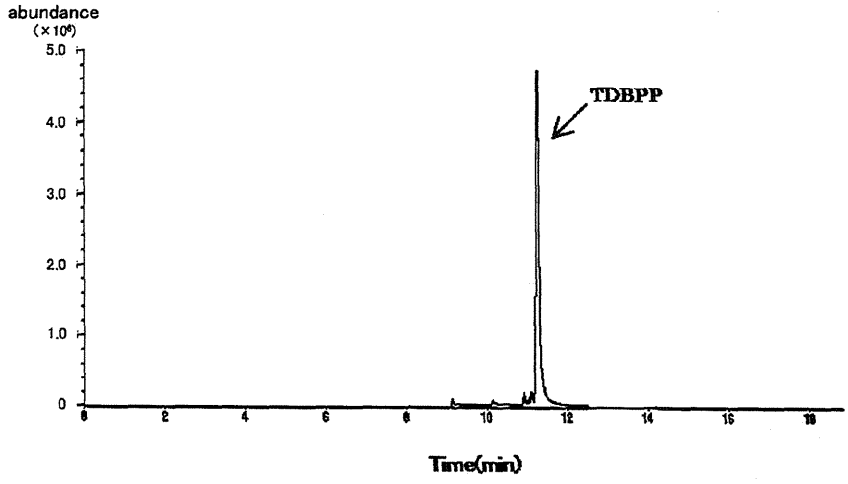

Fig. 2. FPD Gas Chromatogram of TDBPP Using DB-1 Capillary Mega Bore Column

質の除去　繊維製品からは，界面活性剤や脂溶性の共存物質も抽出されてくる．水-シクロヘキサンによる液-液分配後も，これら成分がともにシクロヘキサン層に移行する．そこで，フロリジルカラムによる脂溶性物質の除去法を検討した．50 μg/mL，及び100 μg/mL の TDBPP シクロヘキサン溶液 2 mL をカラムに負荷し，20 mL のヘキサンで洗浄したところ，TDBPP はカラムに保持された．次に，30%エタノール含有ヘキサン 30 mL で溶出したところ，90%以上の TDBPP が回収された．そこで，ヘキサン 20 mL で脂溶性物質を溶出（除去）した後，30%エタノール含有ヘキサン 30 mL で TDBPP を溶出することにした．

なお，フロリジルカラムに負荷する TDBPP 溶液を，シクロヘキサンから酢酸エチル-ヘキサン混液（2：3）に変更し，同様の操作を行ったところ，試料溶液負荷から 20 mL のヘキサンで洗浄するまでの工程で，100, 200 μg 負荷のいずれにおいても約30%の TDBPP の溶出が観察された．

5. 市販防炎加工繊維製品の分析及び添加回収実験　市販防炎加工繊維製品 5 試料を今回構築した方法により抽出・精製した後に，メガボアカラムによる GC/FPD で定量したが，いずれの製品からも TDBPP は検出されなかった．しかし，GC/FPD 測定でいくつかのピークが確認されたことから，様々なリン化合物が含まれている可能性が考えられた．

最も夾雑ピークの多かったカーペットのほかに，カーテン及び枕カバーの 3 試料に対し 20 及び 200 μg の TDBPP を各 3 回添加した添加回収実験を行った（n=3）．その結果を Table 1 に示す．いずれも 100%前後の回収率が得られ，変動係数（CV）も小さく再現性も良好であった．なお，2 μg の添加回収実験も行ったが，夾雑ピーク（共存物質）の影響が大きく，良好な回収率及び再現性は得られなかった．TDBPP の難燃剤としての使用濃度は数%で，加工法によって残存量は異なるが，より高温で処理する練り込み加工でも製品中に数十 ppm は存在する[12]ことから，20 μg でも十分な検出濃度と考えられる．

6. GC/MS 測定の問題点と原因究明　現公定法は，リン化合物として GC/FPD で検出する方法である．そこで，より正確に同定できる GC/MS 法を公定法として導入するための検討を行った．ところが，TDBPP 標準品の分解現象，特に GC のスプリットレス注入口での分解が観察された．TDBPP の標準品 2 種類（和光純薬工業製及び SUPELCO 製 A）をヘキサンに溶解し，GC/MS 測定したところ，クロマトグラム上にいくつかのピークが出現した．そこで，新たに 2 種の TDBPP 標準品（SUPELCO 製 B 及び Fluka 製）を購入して，標準液を調製し測定したところ，この 2 種の標準品でも同様のピークが認められた．Figure 3 に TDBPP 標準（SUPELCO 製 B）溶液の SCAN 測定から得られたトータルイオンクロマトグラム（TIC）及び各ピーク（①-④）のマススペクトルを示す［EI 測定条件(1)］．いずれのピークのスペクトルもマスライブラリー（NIST98 及び Wiley275）から検索した TDBPP（CAS No. 126-72-7）のマススペクトルとは一致しなかった．マスライブラリーは，直接導入法によるスペクトルを登録しているため一致しなかったものと考えられる．最大ピークは，Rt＝15.8 min のピーク④であったが，ピーク①，②，③など保持時間の短いいくつかのピークが観察された．マススペクトルでは，ともに m/z=137（C_3H_5BrO），m/z=201（$C_3H_5Br_2$），m/z=217（$C_3H_5Br_2O$），m/z=257（$C_3H_5Br_2O_2P$）など，TDBPP 由来のプロピル基に

Table 1. Analytical Results and Recovery Rate of TDBPP in Textile Products

Sample No.	Usage	Materials	TDBPP (μg/g)	20 μg/g		200 μg/g	
				Recovery Rate (%)	CV (%)	Recovery Rate (%)	CV (%)
1	Carpet	Acryl 100%	N.D.	97.5	1.5	93.4	0.5
2	Curtain	Polyester 100%	N.D.	104	3.1	100	0.3
3	Curtain	Polyester 100%	N.D.	—	—	—	—
4	Pillow Cover	Cotton 100%	N.D.	118	6.3	105	3.0
5	Night Clothes	Acryl 60% + Cotton 40%	N.D.	—	—	—	—

N.D.: not detected.

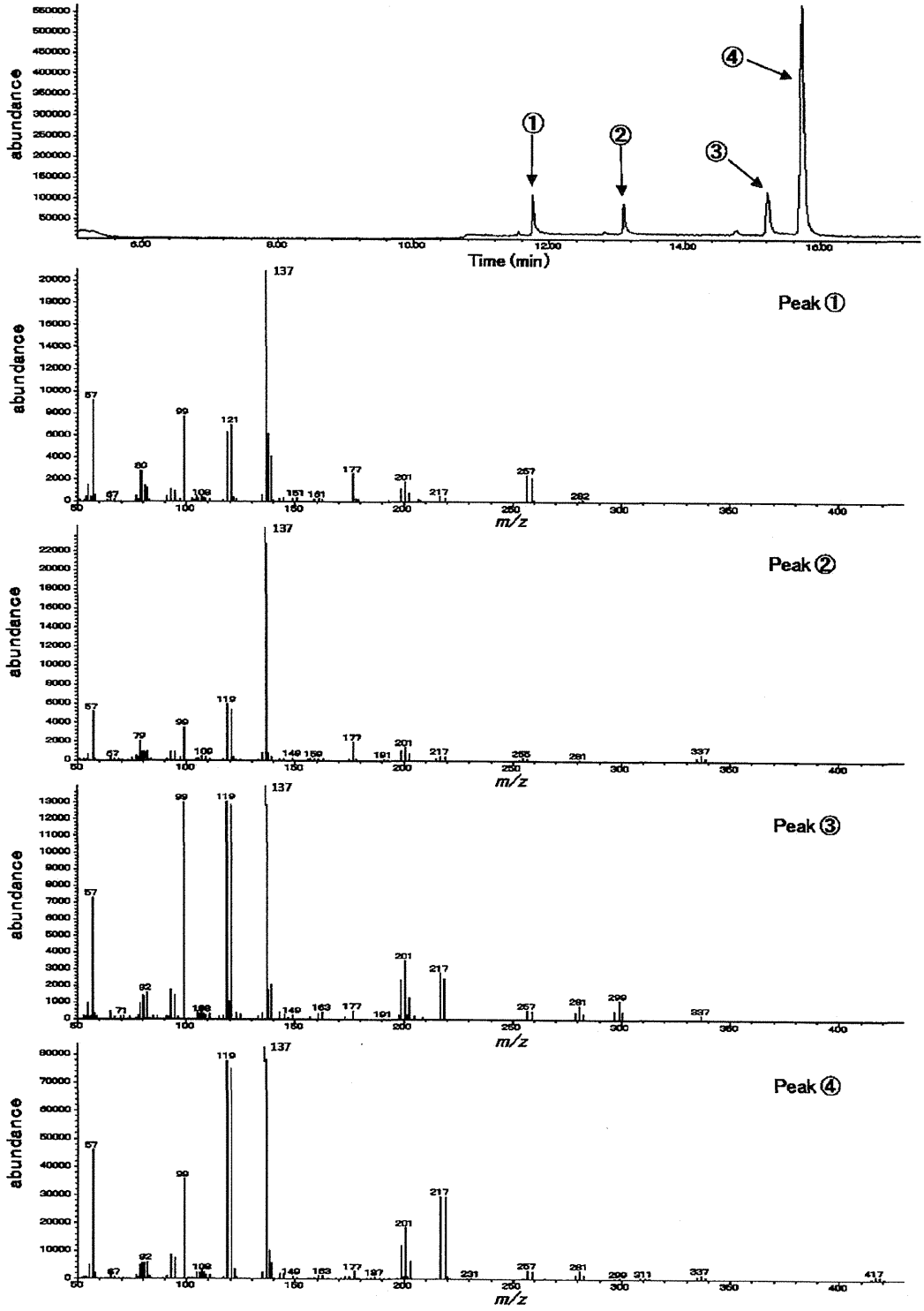

Fig. 3. Total Ion Chromatogram and Mass Spectra (peak①–④) of TDBPP Standard (Supelco B)

①–③: Pyrolysate of TDBPP, ④TDBPP. Operating conditions of GC/MS are given in text. Column temp.: 100°C (2 min)–20°C/min–290°C (6 min). Injection temp.: 290°C, Interface temp.: 290°C. Column: Inert Cap 5 MS/NP (0.25 mmϕ×30 m×0.25 μm).

特徴的なフラグメントイオンが検出された．したがって，GC/MS（EI）だけでは，TDBPP のピークを判定し難かった．TDBPP は，260–300℃で主な熱分解が始まり，Br^- と PO_X を放出するとの報告[13]があることから，これらのピークは GC 分析中に TDBPP が分解したものと考えられた．

そこで，GC/MS と同じキャピラリーカラムで，GC/FPD による標準溶液の測定を行ったところ，同様に TDBPP 以外のリン化合物が検出された．装置・条件が若干異なるものの，主にピーク①–④に相当する保持時間のクロマトグラムが得られた．次に，同じキャピラリーカラム（ DB-5）で，GC/FID による測定も実施したところ，こちらも同様にピーク①–④に相当する保持時間にピークが検出され，炭素鎖を持つ化合物が生成していることが推測された．ところが，同じ標準品についてメガボアカラムを用いた直接導入法による GC/FPD での測定では，メインピーク以外のクロマトグラムは，ほとんど観察されなかった（Fig. 2）．そのため，注入方法の違いによって，注入口での熱分解挙動が変化することが考えられた．そこで，スプリット注入法やクールオンカラム法などの注入口での熱分解の影響が少ない注入法を，同じキャピラリーカラムを用いて検討した．その結果，やはりいくつかのピークが観察されたが，メインピーク④の面積比が相対的に大となった．このように，より適切な注入方法を選択することで，TDBPP の分解が少し改善された．しかし，そのような注入方法を用いても，いくつかの小さいピークが認められることから，注入口での分解だけではなくカラム内での分解の可能性も考えられた．城戸らは，キャピラリーカラムが長いと TDBPP のピークが消失し，短くするほどピークの感度が上昇することを報告している．[14] そこで，注入口やカラムでの熱分解を極力避けるために，注入口には石英ライナーを使用し，カラムの長さを短く（15 m）して測定したところ［EI 測定条件(2)］，ほぼ 1 つのピークとなった（Fig. 4）．このマススペクトルは，メーカー公開の標準品マススペクトルと近似していた．

GC 注入法のみが原因とは断定できないものの，標準品のピークはいくつにも分かれて検出されている．そこで，TDBPP 標準品のピークを確認するために，化学イオン化法（CI 法）による同定を行った［CI 測定条件(1)］．ここでも複数のピークが出現し，ピーク④に相当するピークのマススペクトルには，TDBPP から Br が 1 個脱離し，プロトン付加したと考えられる m/z=619 が，最も強いイオン強度で検出された．m/z=80（Br）と m/z=160（Br_2）が，それにつぐ強いイオン強度で検出された（Fig. 5）．プロトン付加した親イオン（m/z=698）は，

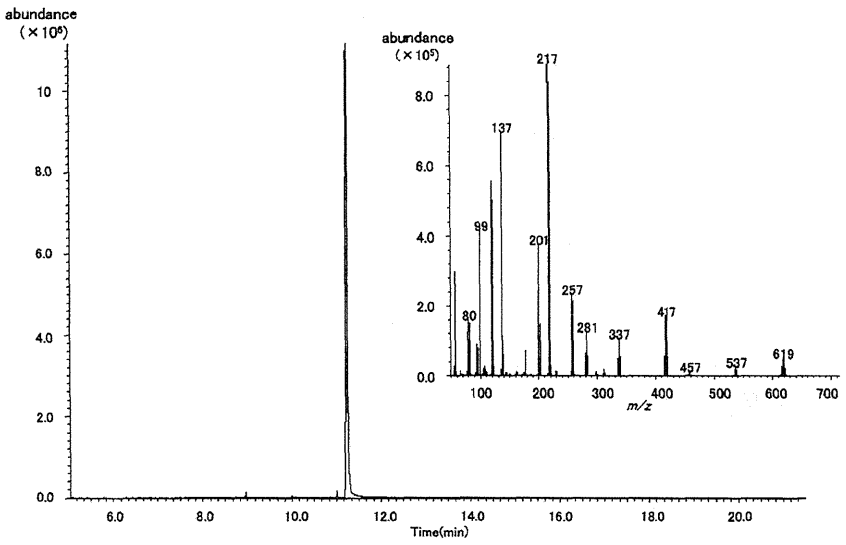

Fig. 4. Total Ion Chromatogram and Mass Spectrum of TDBPP Standard (Supelco B) Using GC/MS (EI) with 15 m Column
Operating conditions of GC/MS are given in text. Column temp.: 100℃ (2 min)–20℃/min–290℃ (6 min). Flow rate: 2 mL/min. Injection temp.: 290℃, Ion source temp.: 160℃, Interface temp.: 250℃. Column: HP-5 (0.25 mmϕ×15 m×0.25 μm).

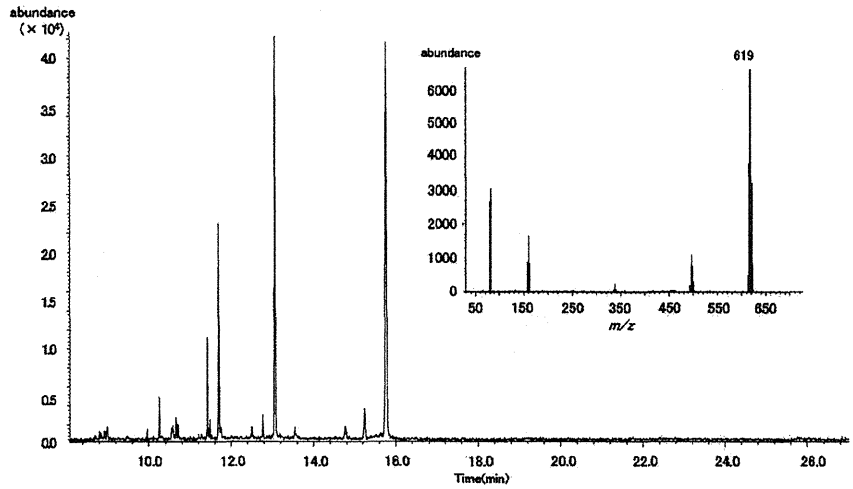

Fig. 5. Mass Spectrum of TDBPP Standard (Supelco B) Using Methane as the Reaction Gas

Operating conditions of GC/MS are given in text. Column temp.: 100°C (2 min)–20°C/min–290°C (6 min). Injection temp.: 290°C, Ion source temp.: 160°C, Interface temp.: 250°C. Column: HP-5MSI (0.25 mmϕ×30 m×0.25 μm).

ほとんど検出されなかった．CI 法でも，イオン源内で TDBPP から Br が脱離することが示唆され，Br が 1 つ脱離したこのピークが TDBPP と推察された．CI 法で，ピーク③に相当するピークのスペクトルは，$C_3H_5Br_2$ が脱離した m/z=496 と，Br が 1 つ脱離した m/z=619 の 2 つが強いイオン強度を示した．ピーク②に相当するピークスペクトルは，$C_3H_5Br_2$ が脱離した m/z=496 と，Br イオンが 2 つ脱離した m/z=536 の 2 つが強いイオン強度を示した．ピーク①に相当するピークスペクトルは，Br が 3 つ脱離した m/z=457 が強いイオン強度を示した．ピーク③，②，①も m/z=80 (Br) と，m/z=160 (Br_2) のイオンが，比較的強い強度で検出された．

さらに，反応ガスにイソブタンを用いて，異なる GC/MS 装置（日本電子製）により CI 測定を行ったところ［CI 測定条件(2)］，感度は低いものの④に相当するピークから親イオンが検出され (Fig. 6)，ピーク④は TDBPP 標準品のピークと考えられた．装置・条件によりイオン源内でも，標準品から Br が脱離する可能性が考えられた．

7. TDBPP 標準品の同定と純度検定　TDBPP 標準品の保証書には，GC/MS スペクトル，FT-IR 及び HPLC（UV-220 nm）のデータが記載されていた．純度検定は，HPLC のデータから 95.5％の含有量との記載があった．メーカー提供の MS スペクトルも親イオンはなかった．そこで，改めて標準品の同定と純度検定を行った．

前述したように，CI 法で標準品の同定を行ったところ，親イオンのみからなるスペクトルは検出できなかった．そこで，標準品を同定するため，高分解能 MS（ESI）の infusion 法で TDBPP 標準溶液を分析した．Positive モードでは $[M+Na]^+$（理論値：m/z=714.57004，実測値：m/z=714.57012，(−0.08 mmu)），Negative モードでは $[M-H]^-$（理論値：m/z=690.57354，実測値：m/z=690.57335，(−0.19 mmu)）と親イオンが検出され，理論値とほぼ同じ値であった．

次に，標準品の（SUPELCO 製 B）の純度検定を行った．前述したように，①-④のピークのマススペクトルからは，m/z=137 (C_3H_5BrO)，m/z=201 ($C_3H_5Br_2$)，m/z=217 ($C_3H_5Br_2O$)，m/z=257 ($C_3H_5Br_2O_2P$) など，TDBPP 由来のプロピル基に特徴的なフラグメントイオンが検出されている．すなわち，TDBPP と同じ炭素数（C9）を持つ等モルの *n*-ノナンを GC/FID で同時測定し，各々のピーク面積比を比較して TDBPP 及び分解産物の含有量を推定することとした．100 μg/mL の TDBPP (SUPELCO 製 B) と等モル数（143 μmol/L）の *n*-ノナンを含有するヘキサン溶液を調製した．その溶液を GC/FID で測定し，各ピーク面積比からピーク①-④に相当する物質の炭素含量を求めた．GC/FID

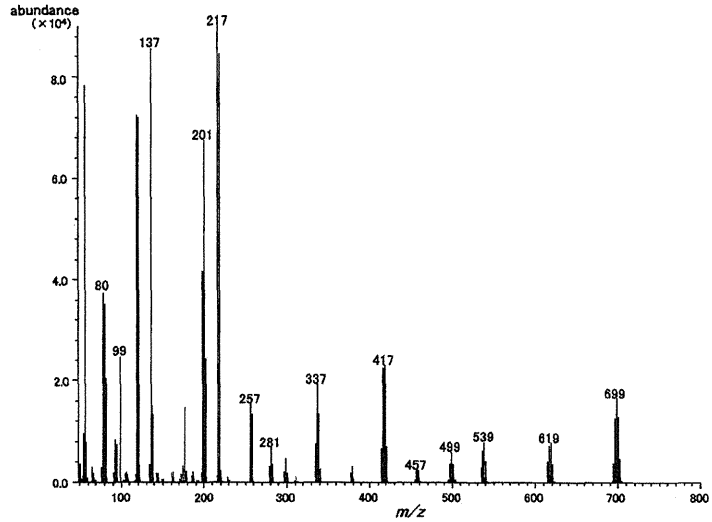

Fig. 6. Mass Spectrum of TDBPP Standard (Supelco B) Using 2-Methylpropane as the Reaction Gas
Operating conditions of GC/MS are given in text. Column temp.: 100°C (2 min)–20°C/min–290°C (6 min). Injection temp.: 290°C, Ion source temp.: 160°C, Interface temp.: 250°C. Column: DB-5MS (0.25 mmϕ×30 m×0.25 μm).

測定条件は，*n*-ノナン測定のため，昇温条件（40°C (4 min)–20°C/min–290°C (8 min)）のみを変更した．*n*-ノナンのピーク面積に対し，TDBPP である④の面積比は 86.5%，①は 8.0%，②は 2.5%，③が 1.8%であった．①–④の合計は 98.8%であり，ほぼ純品であることが分かった．

8. 公定分析法の改定　本研究は，「有害物質を含有する家庭用品の規制に関する法律」により使用禁止されている TDBPP の公定分析法改定を目的としている．ところが，検討過程で TDBPP は熱分解すること，それも GC/MS 装置の注入口，カラム，イオン源内で熱分解が起こることが分かった．つまり，GC/MS だけでは，TDBPP のピークを判定し難く，標準品については別途，7 項で記載したような標準品の同定及び純度検定を行う必要がある．

前述したように GC に適用可能な前処理法を確立した．しかし，測定法（定量・定性）には問題点が残った．今までの結果を踏まえた上で，公定分析法として，以下の方法が候補として考えられる．

(1) GC/FPD による定量及び GC/MS による定性法

現公定法のパックドカラムをメガボアカラムに変更し，直接注入法を用いた GC/FPD 測定で定量する．そして，ピークが検出された場合は，GC/MS (CI, EI) 法を用いてそのマススペクトルで確認する．例えば，EI 法によるピーク④のマススペクトルは（Fig. 3），メーカー提供の標準品マススペクトルと近似している．これをマスライブラリーに登録して，一致率から確認する．CI 法を用いる場合にも親イオンのみは検出できないことから，標準品のマススペクトルと試料ピークのマススペクトルを比較して，一致しているかを確認する．例えば Br が 1 つ脱離したスペクトル（Fig. 5）のピークを TDBPP として同定確認するとよい．

(2) GC/MS (EI) による定量・定性法

注入口に石英ライナー用い，カラムの長さを短く (15 m) することで，ほぼ 1 つのピークとなった [EI 測定法(2)]．このピークのマススペクトルとメーカー提供の標準品マススペクトルは，ほぼ一致した（Fig. 4）．そこで，定量イオンに m/z=217 を，確認イオンに m/z=137 を用いて，5–150 μg/mL の範囲で検量線を作成したところ，R^2=0.99 以上の良好な直線性を示した．検出限界は S/N=3 として 2 μg/mL であった．50 μg/mL 標準溶液を 5 回繰り返し注入したところ，再現性は CV=13.4%であった．再現性のばらつき及び低感度であることなど，少し問題点は残っているものの，定量分析法として適用可能と考えられる．

結　　論

「有害物質を含有する家庭用品の規制に関する法律」により，繊維製品への使用が禁止されている，トリス（2,3-ジブロムプロピル）ホスフェイト（TDBPP）の公定法を改定するために，分析法の検討を行った．

(1) キャピラリーカラムを用いたGC/MS法では，TDBPPが熱分解すること，その現象は，注入口，カラムさらにイオン源でも生じることが分かった．(2) メガボアカラムを用いたGC/FPD測定により，液-液分配及びフロリジルカラム精製で，100％前後の良好な回収率を得る前処理法を確立した．(3) 標準品の同定をESI/MSで，純度検定をGC/FIDで行い，ほぼ100％であることを確認した．(4) 公定分析法としては，①GC/FPD測定後にGC/MSでの同定，②石英ライナー，ショートカラムを用いたGC/MS測定を提案した．

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Cytotoxic and Genotoxic Effects of Silver Nanoparticles on Primary Syrian Hamster Embryo (SHE) Cells

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RESEARCH ARTICLE

Silver-nanoparticles (NPs) have become increasingly common in various applications, raising some safety concerns. In this study, the cytotoxic and genotoxic effects of silver-NPs on primary Syrian hamster embryo (SHE) cells were investigated. Cell viability was assessed using a methyl tetrazolium (MTT) assay, and genotoxic potential was evaluated using a cytokinesis-block micronucleus (CBMN) assay. The results showed that dose-dependent cytotoxicity was induced after 24 h of exposure to silver-NPs. The micronucleation frequency (MNF) also increased significantly in a dose-dependent manner ($P < 0.05$), suggesting that silver-NPs induce genotoxicity. This is consistent with an increased MNF observed in primary SHE cells. The results of cell cycle analysis indicate that the cell cycles became arrested in the $G0/G1$ phase and that the S phase shortened after only 8 h of silver-NP exposure, suggesting that DNA replication had been inhibited, which in turn inhibited further cell proliferation. The rate of late-stage apoptosis increased after 12 h of silver-NP exposure, and both early- and late-stage apoptosis were obviously increased after 72 h of exposure than in controls. This study demonstrated that silver-NPs could induce strong cytotoxicity and significant genotoxicity in primary SHE cells and that this is probably due to silver-NP-induced apoptosis and the inhibition of cell proliferation.

Keywords: Silver-NPs, Primary Syrian Hamster Embryo (SHE) Cells, Cytotoxicity, Genotoxicity, Cell Cycles, Apoptosis.

1. INTRODUCTION

Nanomaterials are seeing increasingly frequent use in commercial applications because of their novel physicochemical properties. However, concerns have arisen regarding the adverse effects of nanomaterials on human health and the environment.[1] Nanomaterials have been shown to induce toxicity in cells, tissues, and organs.[2–7] Of the various nanomaterials, silver-nanoparticles (NPs) are the most commonly used in consumer and industrial products, including medical products and devices, food storage materials, and various health care products. This is largely due to their antibacterial activity.[8–12] Consequently, silver-NPs have also raised the most safety concerns. Silver-NP-induced cytotoxicity and genotoxicity have been observed in many *in vivo* and *in vitro* studies.[13–23]

Many of these studies have suggested that silver-NP treatment can increase DNA damage and chromosome aberrations in various types of cells.[19,21,22,24] However, most of these studies have been based on particular cell lines. One study showed that chromosomal aberrations occurred in 20% of U251 human glioblastoma cells subjected to silver-NP treatment, but also occurred in 16% of untreated cells.[21] In contrast, silver-NP-treated IMR-90 cells, which are normal human lung fibroblasts, showed an aberration rate of 10%, and untreated control cells showed no chromosome aberrations.[21] These findings show that cancer cells have a higher rate of spontaneous chromosome aberration, suggesting that these cells are not suitable for genotoxicity testing; but normal cells have a lower rate of spontaneous chromasone aberration, making these cells are suitable for genotoxicity testing. In the present study, primary Syrian hamster embryo (SHE) cells were used to determine the genotoxic potential of silver-NPs.

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Reactive oxygen species (ROS) induce oxidative damage in DNA. In this way, they play an important role in the cytotoxicity and genotoxicity of nanomaterials.[7, 25–28] The cytokinesis-block micronucleus (CBMN) assay is based on the blocking of cytokinesis by cytochalasin B (Cyt-B), which can express micronuclei produced in response to chromosome breakage or damage to the mitotic apparatus.[29–31] In the present study, a CBMN assay was chosen to assess the genotoxic potential of silver nanoparticles on primary SHE cells.

2. MATERIALS AND METHODS

2.1. Test Materials and Characterization

Silver-NPs (size $\leq$ 100 nm, approx. 99.5%) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.). Silver-NP was dissolved in distilled water and cell culture media and dispersed using ultrasonication for 10 min. The size distribution was determined using dynamic light scattering (DLS). Drops of silver particle solution were placed on a copper net for observation of size morphology using transmission electronic microscopy (TEM). The zeta potentials (ZPs) associated with the silver nanoparticles were also determined to assess their stability in solution and possible interactions with the media using a Malvern Zeta Sizer Nano ZS (Malvern Instruments, Worcestershire, U.K.) operating with Dispersion Technology Software version 5.03 (DTS Nano).

2.2. Chemicals

Thiazolyl blue tetrazolium bromide, 3-(4, 5-dimethylthyl-thiazol-2, 5- diphenyltetrazolium bromide (MTT, $\geq$ 97.5%), cytochalasin B (Cyt-B), and mitomycin C (MMC) were used. The MTT was dissolved in phosphate-buffered saline (PBS, 5 mg/mL), the Cyt-B was dissolved in dimethyl sulfoxide (DMSO, 2.0 mg/mL), and the MMC was dissolved in NaCl solution (10 μg/mL) for use as stock solution. All solutions were sterilized before use by filtration through a 0.2 μm-pore film and stored at −20 °C.

2.3. Animals

Syrian golden hamsters at day 13 of gestation were purchased from Beijing Wei Tong Li Hua Experimental Animal Technology Co., Ltd. (Beijing, China). Animal experiments were conducted in compliance with China's national ethical standards.

2.4. Solutions and Reagents

Wash solution for cell isolation: calcium-magnesium-free Hanks Balanced Salt Solution (CMF-HBSS, Gibco, U.S.) containing 200 U/mL of penicillin and 200 μg/mL of streptomycin (Gibco, U.S.); dissociation solution (prepared on the day of cell isolation): CMF-HBSS containing 1.25% (v/v) of trypsin and 2.5% (v/v) of pancreatin (Sigma, U.S.), 200 U/mL of penicillin, and 200 μg/mL of streptomycin (solution pH was adjusted to 7.0–7.2 before use by adding 7.5% sodium bicarbonate solution); cell isolation media and complete culture media: 500 mL of Dulbecco's Modified Eagle high-glucose medium (DMEM, Gibco, U.S.) containing 130 mL of fetal bovine serum (FBS) (Gibco, U.S.), 13 mL of 200 μM L-glutamine (Gibco, U.S.), and 6.5 mL of 10,000 U penicillin and 10,000 μg/mL streptomycin; cryopreservation media: 85 mL of complete culture media, 15 mL of DMSO (Sigma, U.S.); detachment solution: HBSS containing 0.05% trypsin and 0.53 mM EDTA (Sigma, U.S.).

2.5. Cell Isolation and Culture

Embryo cells were isolated from the embryos of two pregnant hamsters on day 13 of gestation. The hamsters were killed via CO_2 asphyxiation, and the uterine horns were removed and transferred to a 50 mL sterile tube containing ice-cold wash solution. The embryos were removed from the horns and transferred to new Petri dishes containing ice-cold wash solution in a class II biosafety cabinet. About 25 embryos (from two pregnant hamsters) were decapitated, eviscerated, and delimbed, and the remaining tissues were placed in a sterile 100 mL beaker containing 10 mL of wash solution. The tissues were minced into smaller (3 mm) pieces using scissors and then rinsed with 80 mL of wash solution by placing the beaker on a stir plate at low speed for 5 min. Once the tissues had settled, the wash solution was discarded. This rinse step was repeated using dissociation solution (room temperature). Then 50 mL of dissociation solution was added to the flask and slowly stirred for 8 min. The cells were collected, and then 2 mL of cold FBS was added to four 50 mL sterile tubes (on ice). The tissues were allowed to settle and the supernatant (containing dissociated cells) were carefully transferred into the prepared tubes. Then 50 mL of fresh dissociation solution was added to the tissues and stirred for 7 min. The cells were then collected as described above. This dissociation step was performed a total of four times. Cells were collected by centrifugation at 1000 rpm for 5 min and then pooled in 40 mL complete culture media containing antibiotics. The cells were filtered using a 70 μm pore filter (BD, U.S.) and left on-ice until seeding. The viable cells were counted using the trypan blue exclusion method and then seeded (1×10^7 viable cells/T-75 flask) with a complete culture medium containing antibiotics. After incubation at 37 °C and 7% CO_2 for 4 h, the culture media were then replaced with 20 mL of complete culture media without antibiotics. After incubation for another 24 h, cell growth reached about 70% confluence. The culture media were then discarded, and the cells were rinsed twice with 10 mL of CMF-HBSS. Then 1 mL of detachment solution was added to each flask

and allowed to incubate for 2–3 min. Then 10–20 mL of cold complete culture media without antibiotics was added to stop trypsin activity. The cells were pooled in 40–50 mL of fresh complete culture media without antibiotics. The cells were counted and resuspended in cryopreservation media, which were diluted with culture media (final: 7.5% DMSO) to 2.5×10^6 cells/mL. One milliliter of cell suspension was dispensed into cryostorage vials (Nunc), and the cells were frozen in a −70 °C freezer for 24 h, prior to transfer to and storage under liquid nitrogen.

The cells were cultured in DMEM supplemented with 20% FBS, 4 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cultures were maintained in a humidified atmosphere at 37 °C with 7% CO_2.

2.6. MTT Assay

The cytotoxicity of the silver-NPs was assessed using the MTT assay by measuring the optical density of the formazan product. Briefly, 1×10^4 SHE cells (at the fourth passage) were seeded into each well of a 96-well plate and cultured for 24 h, reaching 70% cell confluence. The cells were then exposed to silver-NPs at 0, 2.5, 5, 10, 20, and 40 μg/mL for another 24 h. Before being added to the cells, the silver-NPs were diluted in culture media and ultrasonically treated (ultrasonicator; Beijing Jinxing, China) at 300 W, for 10 min. Each dose group was set for 8-repeats. After exposure, the cells were washed, and a mixture of 20 μL of MTT (5 mg/mL) and 100 μL of non-phenol-red media was added to the cells and incubated for 4 h. The MTT solution was then discarded, and the cells were washed with PBS. Next, 150 μL of DMSO was added to the cells, and the plate was shaken for 10 min. Then, 100 μL of supernatant from each sample was transferred using a multichannel pipette to a new 96-well plate for assay. This transfer decreased the disturbance caused by the remaining silver-NPs to the optical density (OD) detection. The assay was performed using a plate reader at a wavelength of 570 nm, with 630 nm as the reference wavelength. These results are expressed as cell viability relative to untreated control cells.

The modified Karber formulation $\lg IC_{50} = Xm - I(P - (3 - Pm - Pn)/4)$ was used to estimate the 50% inhibitory concentration (IC_{50}) of silver-NPs to SHE cell viability. The following abbreviations were used: Xm, lg max dose; I, lg (max dose/next dose); P, average inhibition rate; Pm, maximum inhibition rate; Pn, minimum inhibition rate.

2.7. CBMN Assay

A CBMN assay was performed using the protocol described below. The primary SHE cells (fourth passage) were seeded at 5×10^5 cells/35 cm in a cell culture dish and let the cells were allowed to grow to 70–80% confluence for 24 h. Then the cells were exposed to 10, 20, and 40 μg/mL of silver-NPs. The silver-NPs were diluted in culture media before being added to the cells. Then they were ultrasonicated for dispersal (ultrasonicator; Beijing Jinxing, China) at 300 W, for 10 min. MMC (0.1 μg/mL) was used as a positive control, and NaCl (50 μL/mL) was used as a negative control. The cells were then cultured for another 24 h. The cells were washed and then cytochalasin-B (final: 3 μg/mL) was added. The cells were cultured for another 18 h. The cells were collected, and the supernatant was discarded. The cells were hypotonically treated with 2 mL of 0.075 M KCl and allowed to sit at room temperature for 5 min. Then 5 mL of fixing solution (methyl alcohol:acetic acid = 3:1) was added, and the mixture was centrifuged immediately (1000 rpm for 5 min). The supernatant was then discarded, and 5 mL of fresh fixing solution was added. The cells were fixed for over 30 min. The fixing solution was then changed again, and the cells were fixed for another 10 min. Cells were dropped onto the center of a slide. The slides were dried at room temperature and stained using 4% Giemsa solution (pH 6.8) for 30 min.

The slides were scored by two experts blinded to the purpose of the experiment at 400× magnification. The micronucleation frequency (MNF, %) was determined for 1000 binucleated cells (BNCs) per culture, and three cultures were established per group.

2.8. Analysis of the Cell Cycle and Apoptosis

The duration of exposure to silver-NPs (4, 8, 12, 24, and 48 h) was established on the basis of the cell cycles of SHE cells. In brief, 8×10^5 cells were seeded in a 60 cm cell culture dish and allowed to grow to 70% confluence for 24 h. Then, 20 μg/mL silver-NP solution was added to the culture media, and, after ultrasonic treatment, the media were added to the cells. The cells were harvested after the specified lengths of time. The cells were with PBS and fixed in 75% ice-cold alcohol and stored overnight in a freezer at −20 °C. Then the cells were collected, washed again with PBS, and stained using propidium iodide solution (containing RNase, MultiSciences Biotech Co., Ltd., China) for 30 min, protected from light. The samples were analyzed using a flow cytometer (BD FACSCalibur, Becton Dickson, U.S.). For analysis of apoptosis, 10 μL of Annexin V-FITC (BIOSEA Co., Ltd., China) was added to a 200 μL solution containing fresh cells (1×10^6 cells/mL), protected from light. After 15 min, 300 μL of binding buffer and 5 μL of PI solution were added. The cell samples were then immediately analyzed using a flow cytometer.

2.9. Statistical Analysis

The data are presented as the mean ± SD. All experiments were repeated at least three times. The data were statistically analyzed using t-testing, ANOVA, and Dunnett testing (2-sided) using SPSS version 12. Differences were considered significant at $P < 0.05$.

3. RESULTS

3.1. Characterization of Silver NPs

The morphology of silver-NP in distilled water and in culture medium was observed using TEM (Figs. 1(a) and (b)).

Particle size distribution in distilled water, as determined by DLS, was found to be 9.6% 6.3–20 nm; 33.6% 27.3–51 nm; 27.5% 52–106.2 nm; 29.3% 106.2–629.0 nm (Fig. 1(c)). The particle size distribution in cell culture medium was found to be 4.4% 10.1–59.8 nm;

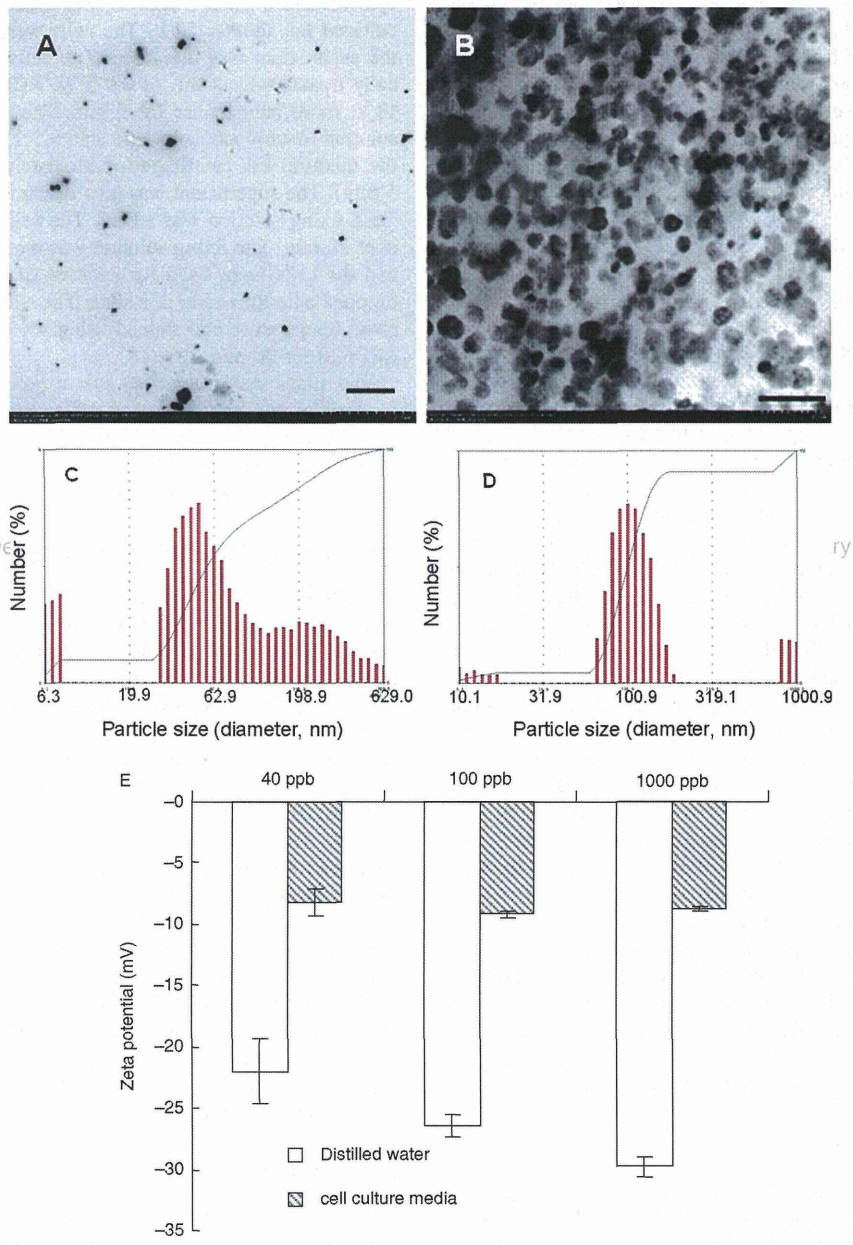

Fig. 1. Characterization of silver-NPs. (a) TEM image of silver-NPs in distilled water (Bar: 1000 nm). (b) Culture media (Bar: 500 nm). (c) Particle size distribution plot of silver-NPs measured by DLS in distilled water. (d) Culture media. (e) Zeta potential of silver-NPs in distilled water and in culture media.