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Chemical analysis and genotoxicological safety assessment of paper and paperboard used for food packaging

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Abstract

This study presents the research on the chemical analysis and genotoxicity of 28 virgin/recycled paper products in food-contact use. In the chemical analysis, paper products were extracted by reflux with ethanol, and analyzed by gas chromatography/mass spectrometry. 4,4′-bis(dimethylamino)benzophenone (Michler's ketone: MK), 4,4′-bis(diethylamino)benzophenone (DEAB), 4-(dimethylamino)benzophenone (DMAB) and bisphenol A (BPA) were found characteristically in recycled products. Seventy-five percent of the recycled paper products contained MK (1.7–12 μg/g), 67% contained DEAB (0.64–10 μ g/g), 33% contained DMAB (0.68–0.9 μg/g) and 67% contained BPA (0.19–26 μg/g). Although, BPA was also detected in virgin paper products, the detection levels in the recycled products were ten or more times higher than those in the virgin products. The genotoxicity of paper and paperboard extracts and compounds found in them were investigated by *Rec*-assay and comet assay. Of the 28 products tested by *Rec*-assay using *Bacillus subtilis*, 13 possessed DNA-damaging activity. More recycled than virgin products (75% against 25%) exhibited such activity, which, of the compounds, was observed in BPA, 1,2-benzisothiazoline-3-one (BIT), 2-(thiocyanomethylthio)benzothiazole, 2,4,5,6-tetrachloro-isophthalonitrile, 2,4,6-trichlorophenol (TCP), and pentachlorophenol. The critical toxicant in one virgin paper product was concluded to be BIT. Eight samples with DNA-damaging activity were also tested by comet assay using HL-60 cells; six induced comet cells significantly (five times or higher than the control) without a decrease of viable cells. TCP, BZ, DEAB, and BIT also caused a slight increase in comet cells. In conclusion, we showed that most recycled paper products contain chemicals such as MK, DEAB, DMAB, and BPA, and possess genotoxicity. However, the levels of the chemicals in the recycled products could not explain their genotoxic effects.

Keywords: Paper; Food packaging; Recycling; Michler's ketone; Bisphenol A; Benzophenone; 1,2-Benzisothiazoline-3-one; *Rec*-assay; Comet assay

1. Introduction

Paper and paperboard are widely used as food packaging materials, frequently in forms adapted to direct contact with foodstuffs. A number of chemicals, such as slimicides, bleaching agents, and inks, are used during the production process. Virgin paper and paperboard products are produced by pulping, bleaching, and treatment processes. Pulping is the process of separating wood into discrete fibers using acids and bases. Recycled paper and paperboard products are produced from used papers such as, newspapers, magazines and milk cartons, and pulped with water and also cleaned and de-inked with surfactants. Bleaching and treatment processes were conducted next. Bleaching is the process of treating with sodium hypochlorite, chlorine dioxide, hydrogen peroxide, etc., to remove lignin and brighten the color of the pulp. In the treatment process, various additives are used, such as slimicides, sizing agent, paper strength agent. Printing is given at the end. The additives used in paper and paperboard food packaging are governed by national regulations. In the United States, the Food and Drug Administration (FDA) has produced its own positive list (FDA, 2003). In Japan, however, no approved control is in general effect concerning the use of additives for food-contact paper packaging.

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Since it has been reported that dioxins are formed by chlorination of lignin in wood (Beck et al., 1988), oxygen is mainly used for bleaching instead of chlorine. However, bleaching agent containing chlorine is still used (Fukuzawa et al., 2002), which may lead to the production of chlorinated compounds. Slimicides are substances used to control bacteria and fungi, which can produce protein and polysaccharide slimes on the finished products if not adequately controlled (Johnsrud, 1997). There is no report as to whether these compounds remain present in the final paper and paperboard products. Although surfactant is used to de-ink recycled fibers, it is reported that ink ingredients remain present in recycled paper and paperboard (Castle et al., 1997).

Few toxicological studies of paper and paperboard food packaging have been published. Fauris et al. (1998) found that both virgin and recycled paper exhibited cytotoxicity in the form of an effect on RNA synthesis rate in human HeLa cells. Baba et al. (1998) examined genotoxicity using *Bacillus subtilis* and found that most recycled paper products for food use, such as cake box, showed DNA-damaging activity. Binderup et al. (2002) report that recycled fibers are more cytotoxic than virgin fibers in normal human skin fibroblasts and that the response in Ah-receptor assay is also more potent.

We investigated 20 standard compounds (Fig. 1) in paper and paperboard which are used in Japan in contact with food. Sixteen of which were made from virgin paper, and remainder from recycled paper. Benzophenone (BZ) and the three types of dialkylaminobenzophenone are used as photoinitiators in UV-cured ink. The 4,4′-bis(dimethylamino)benzophenone (Michler's ketone: MK) is reported to be a potential carcinogen (NCI, 1979a, NTP, 1983, Kitchin and Brown, 1994). As it has been reported that bisphenol A (BPA), which is used as a developer in carbonless paper and thermal paper, is easily chlorinated by sodium hypochlorite, and as chlorinated BPAs have been detected in wastewater from wastepaper recycling plants (Fukuzawa et al., 2001, 2002), five chlorinated BPA congeners were also investigated. BPA and chlorinated congeners have been reported to possess estrogen activities (Fukuzawa et al., 2002). Seven slimicides [5-chloro-2-methyl-4-isothiazolin-3-one (Cl-MIT), 2-(thiocyanomethylthio)benzothiazole (TCMTBT), 1,2-benzisothiazoline-3-one (BIT), 2,4,5,6-tetrachloro-isophthalonitrile (TPN), 1-bromo-3-ethoxycarbonyloxy-1,2-diiodo-1-propene (BECDIP), 3-iodo-2-propyl butylcarbamate (IPBC), and *p*-chlorophenyl-3-iodopropargylformyl (CPIP)], and three chlorinated aromatic compounds [2,4,6-trichloroanisole (TCA), 2,4,6-trichlorophenol (TCP), and pentachlorophenol (PCP)], were additionally investigated. Cl-MIT and TCMTBT have been shown to be mutagenic in bacterial test (BIBRA working group, 1990, Connor et al., 1996), and TPN has been reported genotoxic in comet assay and chromosomal aberration test (Vigreux et al., 1998, Godard et al., 1999). TCMTBT, BIT, TPN, BECDIP, IPBC and CPIP have been reported their allergenic effects (Noda et al., 1998, Shimizu et al., 2000, Yamano et al., 2001). TCP and PCP are reported to be potential carcinogens (NCI, 1979b, NTP, 1989). Moreover, PCP has been reported genotoxic in chromosomal aberration test and sister chromatid exchanges test (NTP, 1989).

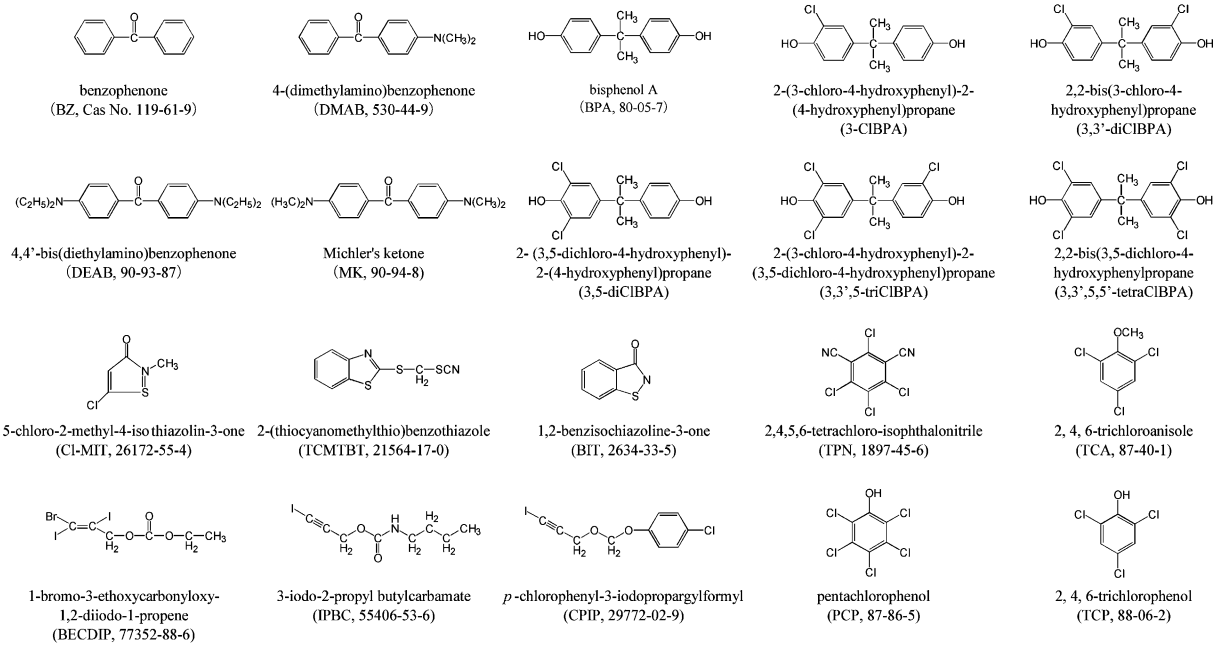

Fig. 1. Structures of tested compounds.

In order to assess the overall safety of paper and paperboard food packaging, genotoxicity test was performed along with chemical analysis. The extracts of paper or paperboard were investigated using two different in vitro toxicity tests: *Rec*-assay is a screening test for mutagenic compounds in which wild-type *B. subtilis* (H17 Rec^+) and its mutant (M45 Rec^-), the recombination-less strain, are used to compare the increased lethal activity of the agent; the assay procedure is simple and inexpensive, and has been used to examine a number of environmental mutagens in a variety of substances such as food additives, pesticides, and metal compounds (Kada et al., 1980, Ozaki et al., 1998, 2002). Comet assay (single-cell gel electrophoresis assay: SCGE assay), which is a good predictor of mutagenic and carcinogenic activity in animals, including humans, detects genomic DNA damage in the form of DNA strand breakage, alkali-labile sites, and incomplete excision repair sites in single cells (Singh et al., 1988), and also DNA degradation due to necrosis or apoptosis (Olive et al., 1993, Fairburn et al., 1996).

2. Materials and methods

2.1. Materials

2.1.1. Solvents and reagents

The following substances were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan), Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Sigma–Aldrich Co. (St. Louis, MO, USA) and Sannopuco (Tokyo, Japan): benzophenone (BZ, >99.5%), 4-(dimethylamino)benzophenone (DMAB, 98%), 4,4′-bis(diethylamino)benzophenone (DEAB, 99%), 4,4′-bis(dimethylamino)benzophenone (Michler's ketone: MK, 98%), bisphenol A (BPA, >95%), 2,4,6-trichloroanisole (TCA, 99%), 2,4,6-trichlorophenol (TCP, >97%), pentachlorophenol (PCP, >99%), 2,4,5,6-tetrachloro-isophthalonitrile (TPN, 100%), bis(trimethylsilyl)trifluoroacetamide (BSTFA), dimethyl sulfoxide (DMSO), and Triton X-100 and RPMI-1640 medium. The standard substances used were ProClin 300 (SUPELCO, U.S.A.) containing 2.66% 5-chloro-2-methyl-4-isothiazolin-3-one (Cl-MIT) for Cl-MIT, Ploxcel PL (100% purity, Zeneca Co., Ltd., Tokyo, Japan) for 1,2-benzisothiazoline-3-one (BIT), TCMTB60 (60% purity, Buckman Laboratories, Tennessee, USA) for 2-(thiocyanomethylthio)benzothiazole (TCMTBT), Omacide-IPBC100 (99.7% purity, Olin Japan Inc., Tokyo, Japan) for 3-iodo-2-propyl butylcarbamate (IPBC), IF-1000S (97.5%, Nagase Chemtex Co., Osaka, Japan) for *p*-chlorophenyl-3-iodopropargylformyl (CPIP), and Sunplus (99.05%, Sankyo Co., Ltd., Tokyo, Japan) for 1-bromo-3-ethoxycarbonyloxy-1,2-diiodo-1-propene (BECDIP). Kindly provided by Prof. Y. Terao were 2-(3-chloro-4-hydroxyphenyl)-2-(4-hydroxyphenyl)propane (3-ClBPA), 2,2-bis(3-chloro-4-hydroxyphenyl)propane (3,3′-diClBPA), 2-(3,5-dichloro-4-hydroxyphenyl)-2-(4-hydroxyphenyl)propane (3,5-diClBPA), 2-(3-chloro-4-hydroxyphenyl)-2-(3,5-dichloro-4-hydroxyphenyl)propane (3,3′,5-triClBPA), and 2,2-bis(3,5-dichloro-4-hydroxyphenylpropane (3,3′,5,5′-tetraClBPA). Bisphenol-A-d_{16} (BPA-d_{16}), used as surrogate for BPA, was purchased from Hayashi Pure Chemical Ind., Ltd. (Osaka, Japan). Naphthalene-d_8, acenaphthene-d_{10}, phenanthrene-d_{10} and chrysene-d_{12} were used as internal standards and purchased from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). All organic solvents, sodium sulfate, and sodium chloride were of analytical reagent grade or higher, and purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Low melting-temperature agarose (SeaPlaque GTG agarose) was purchased from Biowhittaker Molecular Applications (Rockland, ME, USA). Fetal bovine serum (FBS) was purchased from ICN Biomedicals, Inc. (Aurora, OH, USA). Water was treated with a Milli-Q Water Purification System after distillation.

2.2. Samples

Twenty-eight paper and paperboard products used in contact with food were classified into two groups: 16 virgin paper or paperboard products and 12 recycled paper or paperboard products. The products, details of which are given in Table 1, were purchased at a supermarket in Japan in 2001.

2.3. Methods

2.3.1. Gas chromatography/mass spectrometry (GC/MS)

The chromatographic analysis was performed on a Hewlett-Packard 6890 gas chromatograph (Agilent Technologies) equipped with a 5973 mass-selective detector. GC conditions were as follows: column, HP-1MS (0.25 mm i.d. × 30 m, film thickness 0.25 μm, Agilent Technologies); oven temperature was held at 50 °C for 2 min, then raised at 10 °C/min to 280 °C and held for 5 min; injection temperature, 280 °C; carrier gas, He at 1 ml/min; injection volume, 1 μl (splitless). MS conditions were as follows: electron impact ionization mode: ion source voltage, 70 eV; ion source temperature, 220 °C. Quantitation of the analytes was carried out in the selected ion monitoring (SIM) mode after selection of the monitor ions from the full spectra.

2.3.2. Sample preparation method

In order to exclude the influence of the inks used in print, the printed surface of the paper or paperboard was peeled off before extraction (Table 1). To prepare each sample, three examples of the product with the same lot number were purchased and equal amounts of

Table 1
Details of tested samples

Sample no.	Product	Notes
Virgin paper products		
1	Coffee filter	
2	Coffee filter	Non-bleached
3	Cooking paper	
4	Cooking paper	
5	Cooking paper	
6	Cooking paper	
7	Cooking paper	
8	Cup	PE (polyethylene)-coated
9	Dish	
10	Dish	Semi-bleached
11	Flour bag	PE-coated
12	Napkin	
13	Napkin	Semi-bleached
14	Tea bag	
15	Tissue	
16	Fried chicken Wrapping paper	
Recycled paper products		
17	Cake box	Outer layer (printed layer) removed
18	Cardboard box	
19	Confectionery box	Outer layer (printed layer) removed
20	Fried chicken box	Outer layer (printed layer) removed
21	Fried potato box	Outer layer (printed layer) removed
22	Newspaper	
23	Noodle cup	Outer layer (printed layer) removed, PE-coated
24	Noodle cup	Outer layer (printed layer) removed, PP (polypropyrene)-coated
25	Paper under pizza	
26	Pizza box	Outer layer (printed layer) removed
27	Pizza box	Outer layer (printed layer) removed
28	Sandwich box	Outer layer (printed layer) removed

each weighed and mixed together. The samples were cut into pieces (1 cm × 1 cm) and 2–5 g placed in a flask. BPA-d_{16} (0.2 μg/1 g sample) was added and refluxed with 100 ml ethanol for 2 h. After filtration, the ethanol solution was transferred to a separatory funnel together with 500 ml of 2% sodium chloride solution and extracted twice with 50 ml dichloromethane. After evaporation, the combined extract was dehydrated with sodium sulfate and the residue dissolved in *n*-hexane (2 ml/1 g sample). One hundred μl of BSTFA was added to 1 ml of the *n*-hexane solution and the mixture allowed to stand for 1 h to complete trimethylsilylation. The solution was then concentrated to 0.5 ml under a nitrogen stream and made up to 1 ml with *n*-hexane, after which 10 μl of internal standard mixture (10 μg/ml) was added and the solution subjected to GC/MS analysis. Naphthalene-d_8 was used as an internal standard for Cl-MIT and TCA, acenaphthene-d_{10} for TCP, BIT, BZ and IPBC, phenanthrene-d_{10} for TPN, BECDIP, CPIP, PCP, TCMTBT, DMAB and 3-ClBPA, chrysene-d_{12} for 3,3′-diClBPA, 3,5-diClBPA, 3,3′,5-triClBPA, 3,3′,5,5′-tetraClBPA, MK and DEAB. Measurement was carried out using the respective calibration curves, ranging from 0.01 to 1 μg/ml for BZ, BPA, chlorinated BPAs, BIT, TPN, TCA, TCP and PCP, 0.1–10 μg/ml for DMAB, DEAB and BECDIP, and 0.2–20 μg/ml for MK, Cl-MIT, IPBC, CPIP and TCMTBT. Limit of quantitation for each compound was 0.02 μg/g for BZ, BPA, ClBPAs, BIT, TPN, TCA, TCP and PCP, 0.2 μg/g for DMAB, DEAB, BECDIP and MK, 0.4 μg/g for Cl-MIT, IPBC, CPIP and TCMTBT.

2.3.3. Rec-assay

Rec-assay was performed according to the spore method of Kada et al. (1980). A recombinationless strain, M45 *Rec*$^-$ and the wild strain H17 *Rec*$^+$ of *Bacillus subtilis* were used. Briefly, plates were prepared by adding to B-2 (10 g beef extract, 10 g polypeptone, 5 g NaCl, 1000 ml water; adjusted to pH 7.0) agar (1.5%) 2×10^5 spores/ml of strain H17 and M45, preparing 10 ml of the resulting spore agar at 42 °C, pouring it into a level plastic Petri dish (90 mm diameter), and allowing it to solidify well. A paper disc, impregnated with 30 μl of the chemical or sample solution, was then placed on the surface. After 24 h of incubation at 37 °C, the diameters of the inhibition zones were measured. The strains were

kindly provided by the National Institute of Genetics. Chemicals were dissolved in DMSO. To prepare samples, 1 ml of *n*-hexane solution before trimethylsilylation, prepared according to the *sample preparation method*, was dried under a nitrogen stream and dissolved in 100 μl of DMSO.

2.3.4. Comet assay

HL-60 cells originating from a human promyelocytic leukemia cell line were grown in RPMI-1640 supplemented with 7% fetal bovine serum and cultured in an incubator at 37 °C in a 5% humidified CO_2 atmosphere. The cells (1 ml) were grown in 12-well plates at a density of 1×10^6 cells/well for 18 h. The standard compound or sample solution (10 μl) in various concentration in DMSO was then added and incubated. Control cells received an equivalent volume of DMSO alone. After 2 h, a 200 μl aliquot was removed to measure acute cytotoxicity using trypan blue dye exclusion (TBDE) test. The remaining suspension from each well was centrifuged at 4 °C, washed with phosphate-buffered saline (PBS), and suspended in a small volume of PBS. An aliquot of 10 μl of the cell suspension was added to 100 μl of 0.5% low-melting agarose kept at 42 °C and rapidly spread on microscope slides, which were incubated at 4 °C for 10 min to accelerate the gelling of the agarose and then transferred to a prechilled lysis solution (2.5 M NaCl, 100 mM EDTA, pH 7.5, 10% DMSO and 1% Triton X-100, added fresh) for 30 min at 4 °C. A denaturation step was then performed in alkali solution (0.3 M NaOH, 1 mM EDTA) for 20 min, and electrophoresis was conducted for the next 25 min at 25 V, 300 mA. The slides were then transferred to 0.4 M Tris, pH 7.5, for 15 min to neutralize, after which they were fixed in ice-cold 100% ethanol for 5 min and air-dried. For observation, samples were stained with SYBR green (Travigen, Inc., Gaithersburg, MD, USA) diluted 1:10,000 in TE buffer (1 mM EDTA, 10 mM Tris, pH 7.5). All steps described above were conducted under dimmed light to prevent additional DNA damage. The slides were observed at 400× magnification under a confocal laser scanning microscope (Zeiss LSM510, 15 mM argon laser). Images of 200 randomly selected cell samples were classified into four categories for qualitative evaluation (Godard et al., 2002): undamaged cells without tail (undamaged cells: UCs), cells with tiny tail (slightly damaged cells: SDCs), cells with clear tail (damaged cells: DCs) and cells with teardrop-shaped tail (highly damaged cells: HDCs) (Fig. 2).

2.3.5. Statistical analysis

The statistical analysis was performed using the percentage of each type of comet cell, i.e. UC, SDC, DC, and HDC. For assessment, a χ^2 test was performed. A *p*-value of less than 0.01 to the solvent control was considered significant and was noted in the text and the figure.

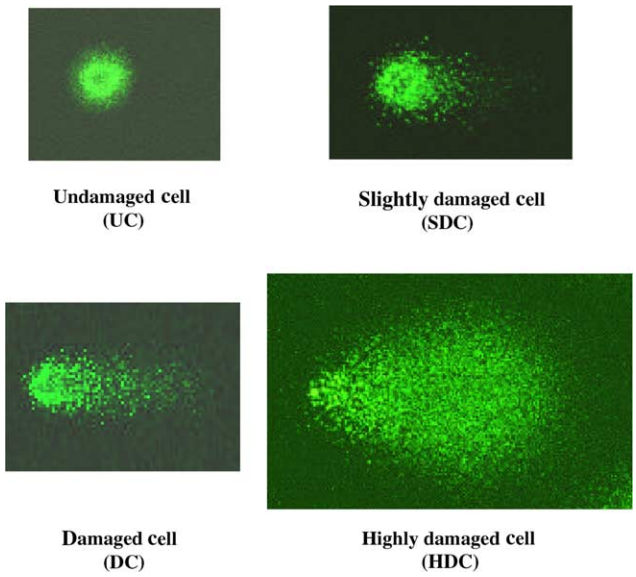

Fig. 2. Photomicrographs of UC, SDC, DC, and HDC observed after comet assay.

3. Results

3.1. Recovery study

A mixture of the target compounds (BZ, BPA, chlorinated BPAs, BIT, TPN, TCA, TCP and PCP: 1 μg, DMAB, DEAB and BECDIP: 10 μg, MK, Cl-MIT, IPBC, CPIP and TCMTBT: 20 μg) was spiked into 5 g of Sample no. 9. The results are shown in Table 2. The recovery values were taken from the average of three trials ($n = 3$). The recovery rates were in the range of 51–128%, and the coefficient of variation (CV) was small.

3.2. Determination

Levels of the 20 standard compounds in the virgin and recycled paper and paperboard products are shown in Table 3. In the 16 virgin products, BZ and BPA were found in many samples (BZ in 8, 0.026–0.09 μg/g; BPA in 13, 0.034–0.36 μg/g), however, detected levels were relatively low. BIT was found in 6 samples (0.070–3.4 μg/g), TCMTBT and TPN in one sample each (1.2 μg/g and 0.03 μg/g, respectively), and TCP in 2 samples (0.074, 0.076 μg/g). Larger amount of chemicals were found in recycled paper products than virgin paper products. In the 12 recycled paper and paperboard products, benzophenones were detected in most samples. BZ was detected in 11 samples (0.032–0.86 μg/g), MK in nine (1.7-12 μg/g), DEAB in eight (0.6–10 μg/g), and DMAB in four (0.68–0.9 μg/g). MK, DEAB and

Table 2
Recovery of 20 compounds from paper products

Compound	Recovery (%) [CV[a] (%)] ($n = 3$)
BZ	97.1 (1.9)
DMAB	100.7 (4.3)
DEAD	112.1 (1.5)
MK	86.8 (0.7)
BPA	128.1 (6.9)
3-C1BPA	68.8 (2.2)
3,3′-diClBPA	78.5 (0.7)
3,5-diClBPA	94.9 (2.0)
3,3′,5-triClBPA	111.0 (2.1)
3,3′,5,5′-tetraClBPA	102.1 (2.2)
Cl-MIT	59.0 (0.6)
BIT	50.6 (3.2)
IPBC	100.0 (5.2)
BECDIP	104.6 (5.1)
CPIP	101.1 (3.8)
TCMTBT	113.9 (6.2)
TPN	72.7 (5.6)
TCA	69.4 (7.1)
TCP	89.4 (2.4)
PCP	93.8 (4.9)

[a] CV: coefficient of variation.

DMAB were detected only in recycled products. BPA was detected in eight samples in a concentration range of 0.19–26 μg/g, and the detection levels in the recycled products were 10 or more times higher than those in the virgin products. BIT was detected in eight in a concentration range of 0.082–0.46 μg/g. However, there was no great difference between virgin and recycled products in the level of detection. PCP was detected in five samples (0.054–0.11 μg/g) only in recycled products. TPN was detected in one sample (0.22 μg/g). Chlorinated BPAs, Cl-MIT, IPBC, BECDIP, CPIP, TCMTBT, TCA, and TCP were not found in any product.

3.3. Rec-assay

The findings for virgin and recycled paper products are shown in Table 4. Of the 28 products tested, 14 produced a killing zone in M45 *Rec*$^-$ and H17 *Rec*$^+$ In 13 of the samples, the killing zone was larger in M45 *Rec*$^-$ than in H17 *Rec*$^+$ but sample no. 3, in which the killing zone was larger in H17 *Rec*$^+$ than in M45 *Rec*$^-$, was concluded to cause cell-growth inhibition in *B. subtilis*. More recycled than virgin paper products (75% against 19% of samples) exhibited DNA-damaging activity, which is in agreement with the findings of Baba et al. (1998), who examined the genotoxicity of methanol extracts of virgin and recycled paper and paperboard food packaging. The results for ten standard compounds detected in virgin or recycled paper products are shown in Table 5. No effective killing action was observed in the benzophenones. BPA, BIT, TCMTBT, TPN, TCP and PCP, on the other hand, produced killing zones in both strain M45 *Rec*$^-$ and strain H17 *Rec*$^+$ larger in the former than the latter, and it was concluded that these substances cause DNA damage in *B. subtilis*. Table 4 also shows the amount of standard compound detected in GC/MS analysis in samples that caused DNA damage in *B. subtilis*. Sample no. 3, which contained 0.51 μg BIT, caused the strongest cell-growth-inhibiting activity and the diameter of the killing zone

Table 3
Levels of 20 compounds in virgin and recycled paper/paperboard food packaging (μg/g)

Sample no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Virgin paper/paperboard food packaging[a]																
BZ	ND	**0.09**	**0.082**	ND	ND	**0.032**	ND	**0.044**	**0.026**	**0.036**	ND	**0.036**	ND	**0.038**	ND	ND
BPA	**0.048**	**0.034**	**0.048**	**0.36**	ND	**0.19**	**0.038**	**0.064**	ND	**0.034**	ND	**0.048**	**0.09**	**0.068**	**0.044**	**0.034**
BIT	ND	ND	**3.4**	**0.07**	**0.082**	ND	ND	ND	ND	ND	ND	ND	**0.14**	**0.31**	**0.092**	ND
TCMTBT	ND	ND	ND	ND	ND	**1.2**	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
TPN	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	**0.03**	ND	ND	ND	ND	ND
TCP	ND	ND	ND	ND	ND	ND	ND	ND	ND	**0.076**	ND	ND	**0.074**	ND	ND	ND

Recycled paper/paperboard food and packaging[b] Sample no.	17	18	19	20	21	22	23	24	25	26	27	28
BZ	**0.1**	**0.52**	**0.12**	**0.054**	**0.26**	**0.86**	ND	**0.032**	**0.034**	**0.69**	**0.066**	**0.15**
DMAB	**0.68**	ND	ND	ND	**0.7**	ND	ND	ND	ND	**0.8**	ND	**0.9**
DEAB	ND	**1.7**	**0.74**	**0.72**	**4.8**	**10**	ND	ND	ND	**3.1**	**0.9**	**0.64**
MK	**1.7**	**12.0**	**4.4**	**4.8**	**2.8**	**4.6**	ND	ND	ND	**8.1**	**5.1**	**3.9**
BPA	ND	**2.0**	**0.89**	**2.4**	**0.74**	**0.19**	ND	ND	ND	**1.3**	**0.37**	**26**
BIT	ND	**0.12**	**0.14**	**0.082**	**0.41**	**0.088**	ND	ND	ND	**0.094**	**0.46**	**0.37**
TPN	ND	ND	ND	**0.22**	ND	ND	ND	ND	ND	ND	ND	ND
PCP	ND	**0.11**	**0.11**	**0.068**	ND	ND	ND	ND	ND	**0.078**	ND	**0.054**

[a] DMAB, DEAB, MK, chlorinated-BPAs, Cl-MIT, IPBC, BECDIP, CPIP, TCA and PCP were not detected in any sample.
[b] Chlorinated-BPAs, Cl-MIT, IPBC, BECDIP, CPIP, TCMTBT, TCA and TCP were not detected in any sample.

Table 4
Rec-assay of virgin/recycled paper products

	Sample no.	Diameter of killing zone (mm)			Amount of compound[a] detected in GC/MS analysis (per disc: μg)
		M45	H17	M45–H17	
Virgin paper products	1	–[b]	–[b]	0.0	
	2	–[b]	–[b]	0.0	
	3	19.0	21.0	−2.0	BPA (0.0072), BIT (0.51)
	4	–[b]	–[b]	0.0	BPA (0.054), BIT (0.011)
	5	–[b]	–[b]	0.0	BIT (0.012)
	6	–[b]	–[b]	0.0	BPA (0.029), TCMTBT (0.18)
	7	–[b]	–[b]	0.0	BPA (0.0057)
	8	11.5	10.5	1.0	BPA (0.0096)
	9	14.0	12.0	2.0	
	10	10.0	–[b]	2.0	BPA (0.0051), TCP (0.011)
	11	12.0	9.5	2.5	TPN (0.0045)
	12	–[b]	–[b]	0.0	BPA (0.0072)
	13	–[b]	–[b]	0.0	BPA (0.014), BIT (0.021), TCP (0.011)
	14	–[b]	–[b]	0.0	BPA (0.010), BIT (0.047)
	15	–[b]	–[b]	0.0	BPA (0.0066), BIT (0.014)
	16	–[b]	–[b]	0.0	BPA (0.0051)
Recycled paper products	17	–[b]	–[b]	0.0	
	18	12.0	10.5	1.5	BPA (0.30), BIT (0.018), PCP (0,017)
	19	11.0	10.5	0.5	BPA (0.13), BIT (0.021), PCP (0.017)
	20	–[b]	–[b]	0.0	BPA (0.36), BIT (0.012), TPN (0.033), PCP (0.010)
	21	11.0	–[b]	3.0	BPA (0.11), BIT (0.062)
	22	10.0	–[b]	2.0	BPA (0.029), BIT (0.013)
	23	15.0	12.0	3.0	
	24	13.5	12.0	1.5	
	25	13.0	10.5	2.5	
	26	11.5	9.5	2.0	BPA (0.20), BIT (0.014), PCP (0.012)
	27	9.5	–[b]	1.5	BPA (0.056), BIT (0.069)
	28	–[b]	–[b]	0.0	BPA (3.9) BIT (0.056), PCP (0.0081)

[a] Compounds causing damage in *B. subtilis* shown.
[b] No growth inhibition observed.

was almost the same as at BIT concentration of 0.6 μg/disc, leading to the conclusion that the component in Sample no. 3 which caused cell-growth-inhibiting activity was BIT. Some samples also contained 0.011–0.021 μg/disc, but BIT caused cell-growth-inhibiting activity at concentrations of 0.3 μg/disc and above. Some samples contained BPA, BIT, TCMTBT, TPN and PCP, but these compounds showed no cell-killing or DNA-damaging activity at the doses contained in the paper samples.

3.4. Comet assay

The eight samples which possessed DNA-damaging activity and the chemicals contained in them were tested by comet assay and TBDE test. The results for the paper extracts and the positive control (MNNG) are shown in Fig. 3. MNNG showed dose-dependent significant increases in SDCs, DCs, and HDCs without decrease in the percentage of viable cells, indicating that it is a typical genotoxin. Non-genotoxins at cytotoxic concentrations may induce DNA migration in some cell lines in the comet assay (Storer et al., 1996, Kiskinis et al., 2002). Accordingly, samples or standard compounds which increased the comet-cell count significantly at concentrations where cell viability was maintained at 75% or above were concluded to be genotoxins (Henderson et al., 1998). Sample no. 8 induced a significant increase of SDC and HDC at 2.5 and 5 mg/ml, without decrease in the percentage of viable cells. Decrease of the percentage of viable cells was observed only at the highest dose. In a similar manner, increase of SDC and HDC was observed in sample nos. 9, 10, 18, 22 and 27 with higher than 75% cell viability. Sample no. 21

Table 5
Rec-assay of 10 compounds

Compound	μg/disc	Diameter of killing zone (mm)		
		M45	H17	M45–H17
BZ	1000.0	–[a]	–[a]	0.0
DMAB	1000.0	–[a]	–[a]	0.0
DEAB	1000.0	–[a]	–[a]	0.0
MK	1000.0	–[a]	–[a]	0.0
BPA	250.0	26.0	22.0	4.0
	100.0	19.0	13.0	6.0
	6.0	–[a]	–[a]	0.0
BIT	6.0	26.0	23.0	3.0
	3.0	22.0	21.0	1.0
	0.6	17.0	16.5	0.5
	0.3	10.5	9.5	1.0
	0.06	–[a]	–[a]	0.0
TCMTBT	6.0	20.0	16.0	4.0
	3.0	12.0	9.0	3.0
	0.6	–[a]	–[a]	0.0
TPN	6.0	30.0	24.0	6.0
	3.0	26.0	21.0	5.0
	0.6	–[a]	–[a]	0.0
TCP	100.0	21.0	15.0	6.0
	50.0	13.0	10.0	3.0
	6.0	–[a]	–[a]	0.0
PCP	6.0	19.0	12.0	7.0
	3.0	10.0	–[a]	2.0
	0.6	–[a]	–[a]	0.0
AF2[b]	0.03	20.0	13.0	7.0
DMSO	–	–[a]	–[a]	0.0

[a] No growth inhibition observed.
[b] Positive control: 2-(2-furyl)-3-nitro-2-furyl)acrylamide.

increased HDC slightly at the highest dose, however, there was no significance. Sample no. 24 did not increase the level of comet cells within the dose which cell viability was maintained. Of the eight paper products tested, 6 samples (nos. 8, 9, 10, 18, 22 and 27) were judged positive and two negative (nos. 21 and 24). No difference was observed between virgin and recycled paper products in the proportion of positive samples. The results for the eight standard compounds detected in virgin or recycled paper extracts are shown in Fig. 4. The setting of maximum concentrations for these standard compounds was based on cytotoxicity and solubility. MK and DEAB were tested at up to 25 μg/ml, as dissolution was not complete at 50 μg/ml. TCP, BIT, DEAB and BZ were judged positive because they increased comet-cell count significantly without cytotoxicity. TCP induced HDC significantly at 50 μg/ml (1.1% in the control to 16.3%) at cell survivals of 78%, BIT induced HDC significantly at 1 μg/ml (0.8 to 9.6%) at cell survivals of 90%, DEAB induced SDC significantly at 25 μg/ml (7.5 to 20.5%) at cell survivals of 95%, and BZ induced HDC significantly at 10 μg/ml (0.8% to 8.9%) at cell survivals of 87%. Most of the standard compounds, except DEAB and MK, induced a decrease in viable cells. BIT was found to be particularly toxic, with 5 μg/ml causing complete annihilation of the cell population. However, no standard compound exhibited comet-cell induction or toxicity at the dose contained in the paper extracts. Since the toxicity of some compounds may be enhanced when combined, two different reconstitution tests were carried out. In the first, standard compounds were mixed at the maximum dose contained in the paper extracts (Mixture-1). In the second, 1/10 of the EC_{50} dose of each standard compound was used (Mixture-2). MK and DEAB were mixed at the maximum dose which was fully soluble. The results are shown in Fig. 5. In Mixture-1, HDC levels increased and viability decreased slightly. The observed effect was much weaker than in most of the paper samples tested, indicating that there are unknown toxicants in the paper extracts. In Mixture-2, an increase in HDC levels was induced in a dose-dependent manner and was accompanied by a decrease in viable-cell count. These effects appeared to be only additive, and not synergic.

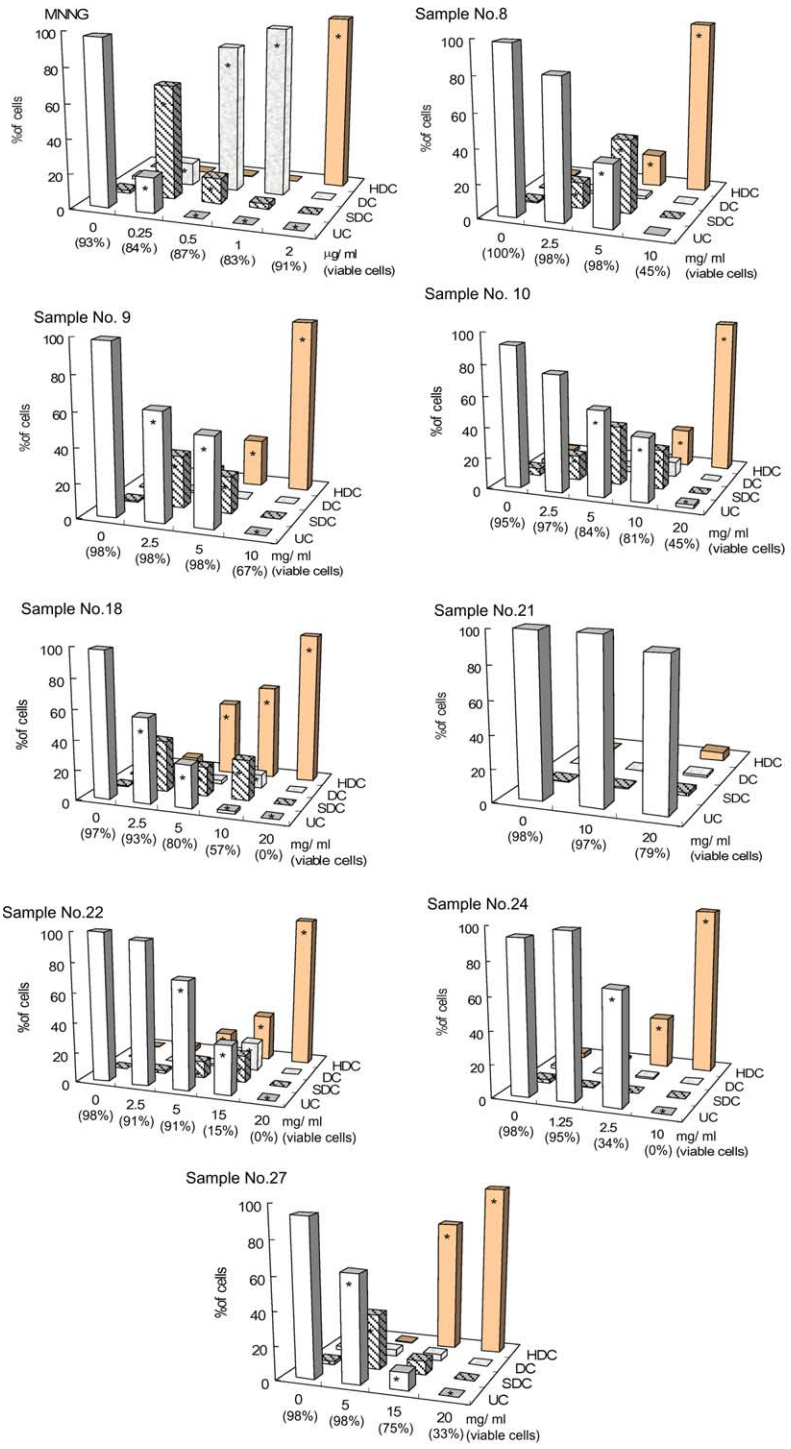

Fig. 3. Comet assay and trypan blue dye exclusion (TBDE) test in HL-60 cells exposed to paper extracts. The percentage of comet cells in each treatment based on random counting of 200 cells is classified into four grades: undamaged cells (UC), slightly damaged cells (SDC), damaged cells (DC) and highly damaged cells (HDC). $^{*}p < 0.01$, compared to solvent control by χ^2 test.

4. Discussion

In the chemical analysis, higher amount of chemicals were found in recycled paper products than virgin paper products. MK, DMAB, and DEAB, which are used as photoinitiators in UV-cured ink (NCI, 1979a, Castle et al., 1997, HSDB, 1992), were detected only in recycled products, indicating that the likely origin is recycled fiber. MK is thought to be a carcinogen (NCI, 1979a, NTP, 1983, Kitchin and Brown, 1994), and the industry in areas including Japan and the European Community has voluntarily placed it on a list of substances not

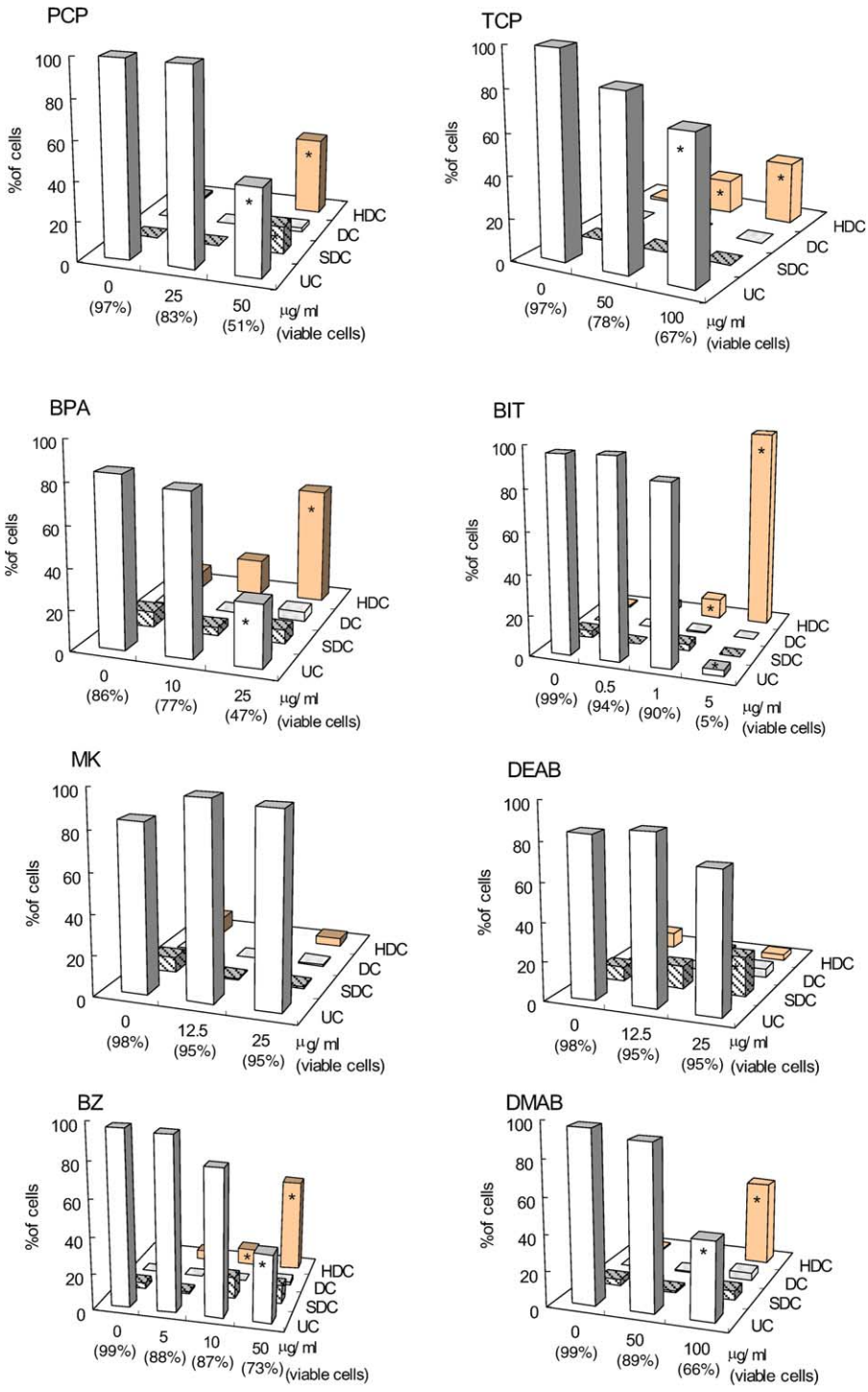

Fig. 4. Comet assay and trypan blue dye exclusion (TBDE) test in HL-60 cells exposed to standard compounds. The percentage of comet cells in each treatment based on random counting of 200 cells is classified into four grades: undamaged cells (UC), slightly damaged cells (SDC), damaged cells (DC) and highly damaged cells (HDC). $^{*}p < 0.01$, compared to solvent control by χ^2 test.

recommended for use in printing ink to be used on paper or paperboard food packaging (Japan Printing Ink Makers Association, 1999; CEPE, 2001). However, it is not clear whether the above compounds are in current use in Japan or other countries in inks for uses other than food packaging. Castle et al. (1997) report that MK and DEAB were detected in 26% (0.1–1.6 μg/g) and 4% (0.2–0.7 μg/g) of 121 paper or paperboard food packaging samples, respectively. They also analyzed the migration level of MK to foods, but found no measurable migration. However, the levels detected in our study were 5–10 times higher. In our study, the printed

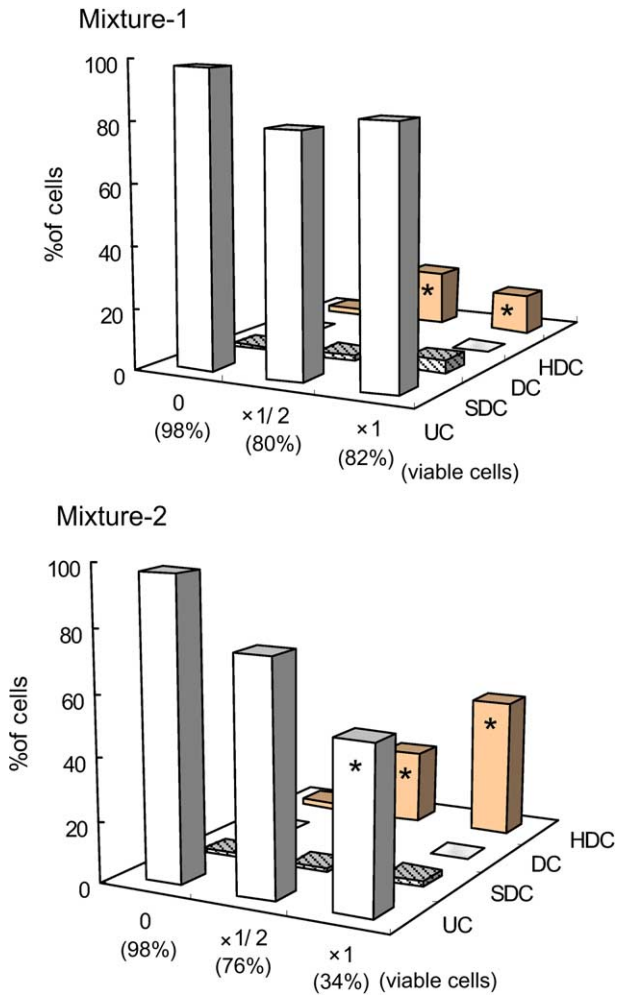

Fig. 5. Comet assay and trypan blue dye exclusion (TBDE) test in HL-60 cells exposed to standard compound mixture. The percentage of comet cells in each treatment based on random counting of 200 cells is classified into four grades: undamaged cells (UC), slightly damaged cells (SDC), damaged cells (DC) and highly damaged cells (HDC). Mixture-1: (BIT) 0.8 μg/ml; (BZ) 0.6 μg/ml; (BPA) 1.6 μg/ml; (DEAB) 2.0 μg/ml; (DMAB) 0.2 μg/ml; (MK) 2.0 μg/ml; (PCP) 0.02 μg/ml and (TCP) 0.02 μg/ml. Mixture-2: (BIT) 0.3 μg/ml; (BZ) 5.0 μg/ml; (BPA) 2.0 μg/ml; (DEAB) 12.5 μg/ml; (DMAB) 14.0 μg/ml; (MK) 12.5 μg/ml; (PCP) 7.0 μg/ml and (TCP) 18.0 μg/ml. $^{*}p < 0.01$, compared to solvent control by χ^2 test.

surface of the paper sample, except newspaper, was peeled off before extraction. The levels found in the newspaper was of the same order of magnitude as other paper products, indicating that inks in newspaper do not contribute to these chemicals. To clarify the source of these substances, further study is required, such as investigation on the level in inks used in paper products not only for food packaging, because various used paper products are used as a material of recycled fibers.

BZ was detected in both virgin and recycled products, but at higher levels in the latter. Johns et al. (1995) reported that the level of BZ in printed paper and paperboard was 22,500–71,000 μg/cm^2 and that 4.6–73% of the BZ migrated to food under microwave heating; since we removed the printed surface to exclude the influence of inks, the level of BZ was lower in the present study. Our results did nevertheless suggest a similar pattern of BZ detection in recycled paper and paperboard products. As with MK, DMAB, and DEAB, the presence of BZ may be due to recycled fiber. The origin of BZ in the virgin products is not clear.

BPA was found in both virgin and recycled paper products, the detection levels in the recycled products were 10 times or higher than those in the virgin products. This finding agrees with reported study (Vinggaard et al., 2000). Also the levels of BPA in recycle products were in good agreement with Vinggaard et al. (2000), who examined the kitchen towels made from recycled fibers. BPA is used as a developer in thermal and carbonless paper production and has been detected in wastewater from wastepaper recycling plants (Fukuzawa et al., 2001, 2002). The origin of BPA detected in recycled paper is presumed to be a contaminant in the recycled fiber. Since a low-dose effect of BPA on estrogenicity has been reported (Rubin et al., 2001, Schonfelder et al., 2002), further study is necessary to examine migration into food simulants or actual foods. BPA is easily chlorinated by sodium hypochlorite and chlorinated BPAs have also been detected in wastewater from wastepaper-recycling plants (Fukuzawa et al., 2001, 2002). Chlorinated BPAs were not found in any sample, suggesting that bleaching agent containing chlorine was not used in the production of any of the samples tested.

There was no great difference between virgin and recycled products in the level of detection of the antimicrobial agent BIT, which is used not only as slimicide but also as fungicide in printing ink, paint, and adhesive (Nakashima et al., 2000). BIT was detected in virgin and recycled products, regardless of material type or whether the surface was printed. This suggests that BIT used as slimicide may remain present in the product. BIT was detected at a particularly high concentration in Sample no. 3, to which it may have been added with antimicrobial intent.

TCMTBT and TPN, which also possess antimicrobial effect as BIT, were detected in few virgin or recycled products. TCMTBT are used as a slimicide and it has been detected in recycled paper mill process water (Rigol et al., 2004). PCP was detected only in recycled products. PCP has been used in large quantities, mainly as a wood preservative. It has also found minor use as a herbicide, defoliant, bactericide and molluscicide. However, its use in agriculture has been restricted in many countries (IARC, 1991). As PCP was not found in any virgin products, it is assumed that recycled products were contaminated from the usage of raw material which PCP was used as a preservative of wood or slimicide of pulp. Diserens (2001) has been reported that

PCP was found in the wood of pallets, containers and in cardboard, and moreover, fruits may be contaminated during storage in wooden crates.

Paper extracts and standard compounds found in them were tested by *Rec*-assay, and more recycled than virgin products (75% against 25%) exhibited DNA-damaging activity. The component in Sample no. 3 which caused cell-growth-inhibiting activity was concluded to be BIT. The eight samples which possessed DNA-damaging activity were tested by comet assay, and six of the eight paper samples induced comet cells. These samples increased the level of SDCs and HDCs, but not DCs. The typical positive control, MNNG, showed dose-dependent significant increases in SDCs, DCs, and HDCs. However, most of the standard compounds tested, such as BPA and PCP, induced mostly HDCs, with decrease in viable-cell count. These differences seem to depend on the toxicity of the compounds. The comet assay is claimed to specifically detect apoptotic cells through the presence of HDCs with lethal effects, meaning that the induction of HDCs by most of the standard compounds tested, including BPA and PCP, was caused by apoptosis. This finding agrees with reported study (Lee et al., 2003) which BPA was evaluated by comet assay and apoptotic and necrotic cells were detected with flow cytometry. MNNG, an alkylating agent, induced comet cells without decrease in viable cells, which was caused solely by DNA damage. In the case of paper samples, an increase in SDCs indicates that some genotoxic components are present. However, when the concentration was raised, no DCs were induced, but HDCs with lethal effects were, indicating that some weak genotoxins and cytotoxins are present in a mixture in these paper samples. These unidentified toxins were not able to explain with standard compounds in paper extracts, and by the reconstitution tests.

In the FDA regulation on indirect food additives used in paper and paperboard, solvents, time and temperature for extraction, are determined by the type of food and conditions which is intended to use. Water, *n*-heptane, or 8% or 50% ethanol are used as food simulants. In the present study, paper samples were refluxed with ethanol (Baba et al., 1998, Vinggaard et al., 2000, Binderup et al., 2002). This extraction procedure is quite severe to give information on which possible toxic compounds could be found in the samples. In order to determine the safety of recycled paper products, more studies such as migration test into food simulants or foodstuff is needed.

A comparison of the results for standard compounds obtained by *Rec*-assay and comet assay is shown in Table 6, along with the findings of previously reported in vitro and in vivo genotoxic and carcinogenic studies. BZ has been reported to be non-genotoxic and non-carcinogenic in NTP studies (NTP, 2000a,b). In the present study also, it was negative in *Rec*-assay, and although it was positive in comet assay, the genotoxicity was relatively weak. DMAB was negative in both assays, while DEAB was negative in *Rec*-assay and positive in comet assay. There have been few studies of the toxicity of these substances, but since both have been found in many recycled paper products, such an examination would seem appropriate. MK has been reported as a genotoxin in almost all in vitro genotoxicity assay such as Ames test, sister chromatid exchange test, and chromosome aneuploidy test (Dunkel and Simmon, 1980; Parodi et al., 1982; Lafi et al., 1986). Moreover, its carcinogenicity in rodent has been also reported (NCI, 1979a; NTP, 1983; Kitchin and Brown, 1994). However, it was not judged genotoxic in the present study. Because of its poor solubility, a high concentration could not be obtained in our assay, and the difference may therefore be due to the relatively low tested dose. Genotoxicity and carcinogenicity of BPA has been reviewed by Haighton et al. (2002), and it has been reported as a non-genotoxin and non-carcinogen in most studies, but as a genotoxin in chromosome aberration test and cell transformation embryo cell transformation assay. In the present study, it was clearly positive in *Rec*-assay but negative in comet assay, indicating that it may possess some genotoxicity. TCP is a reported carcinogen (NCI, 1979b), but is negative in Ames test (Kinae et al., 1981). *Rec*-assay and comet assay, however, was useful to detect its genotoxicity. PCP has been reported genotoxic and carcinogenic (NTP, 1989), but was positive in *Rec*-assay and negative in comet assay in the present study. Pavlica et al. (2001) meanwhile report that significant increase in DNA damage is observed after exposure to PCP. This disagreement may be caused by differences in the cell line and exposure time. In the present study, HL-60 cells were exposed to PCP for 2 h, whereas Pavlica et al. (2001) treated haemocytes of zebra mussel for 7 days. These results indicate that genotoxicity of PCP might be affected by metabolism. However, further study, such as comet assay conducted with the presence of S9mix (rat liver homogenate containing microsomal enzymes plus cofactors), is required to prove it. TCMTBT and TPN were evaluated only in *Rec*-assay and judged as positive. Our results agree with reported studies. TCMTBT has been shown to be mutagenic in bacterial test (BIBRA working group, 1990), and TPN has been reported genotoxic in comet assay and chromosomal aberration test (Vigreux et al., 1998, Godard et al., 1999).

We conducted both GC/MS analysis and toxicological tests on the paper extracts and on 20 selected standard compounds. The toxicant in one virgin paper product was concluded to be BIT, but those in other paper samples remain unidentified. Now that the toxicity of paper and paperboard food packaging has been confirmed in several studies (Fauris et al., 1998, Baba

Table 6
Comparison between *Rec*-assay and comet assay in standard compounds

	Genotoxicity		Reported studies	
	Rec-assay	Comet assay	In vitro genotoxicity studies	In vivo carcinogenic studies
BZ	–	+	Negative in Ames test (TA98, TA1535, TA1537)[a]	Negative in micronucleus test in B6C3F1 mice[b] No induction of carcinoma in Swiss mice[c]
DMAB	–	+		
DEAD	–	+		
MK	–	–	Induction of sister chromatid exchanges in mouse lymphocytes[d] Induction of chromosome aneuploidy in Chinese hamster cells[h] Positive in Ames test (TA1538)[i] Negative in Ames test (TA1538, TA100)[j,k]	Induction of hepatocellular carcinoma in B6C3Fl mice and F344 rats[e,f,g]
BPA	+	–	Negative in most tests (Ames test, mammalian cell gene mutation assay, chromosome aberration test, etc.[l] and comet assay[m]) Positive in some tests (chromosome aberration test, Syrian hamster embryo cell transformation assay, etc.)[l]	No induction of carcinoma in B6C3F1 mice[l]
BIT	+	+	Negative in Ames test (TA98)[n]	
TCP	+	+	Negative in Ames test (TA98, TA100, TA1537)[o]	Induction of lymphoma and leukemia in F344 rats[p] Induction of hepatocellular adenoma and carcinoma in B6C3F1 mice[p]
PCP	+	–	Induction of chromosal aberrations and sister chromatid exchanges in CHO cells[q]	Induction of hepatocellular adenoma and carcinoma in B6C3F1 mice[q,r] Positive in comet assay[s]

[a] NTP (2000a).
[b] NTP (2000b).
[c] Stenback and Shubik (1974).
[d] Parodi et al. (1982).
[e] NCI (1979a).
[f] NTP (1983).
[g] Kitchin and Brown (1994).
[h] Lafi et al. (1986).
[i] Dunkel and Simmon (1980).
[j] Scribner et al. (1980).
[k] McCarthy et al. (1983).
[l] Haighton et al. (2002).
[m] Lee et al. (2003).
[n] Riggin et al. (1983).
[o] Kinae et al. (1981).
[p] NCI (1979b).
[q] NTP (1989).
[r] Ninety percent pure preparation of PCP was used. Typical impurities included were such as tri- and tetrachlorophenol and hexachlorobenzene.
[s] Pavlica et al. (2001).

et al., 1998, Binderup et al., 2002), it is essential to identify the toxicants involved. Bacterial contamination of paper and paperboard used in food packaging has been reported, with aerobic spore-forming bacteria belonging to the genera *Bacillus* as the main contaminants (Vaisanen and Salkinoja-Salonen, 1989; Vaisanen et al., 1991; Pirttijarvi et al., 1996). As some of these strains are enterotoxic, they may be connected to the unidentified toxicants. Many kinds of slimicide are used to control bacteria and fungi. Seven were analyzed in the present study, but many others may be in use, as slimicides are changed periodically to prevent bacterial resistance. Further research is required to determine the DNA-damaging or cytotoxic compounds present in the paper products. Given that DNA-damaging activity was observed mainly in recycled paper products, it seems reasonable to consider that the genotoxins observed in the *Rec*-assay originate from chemicals used in the

recycling process, or from contaminants, chemical or microbial, present in recycled fiber. To clarify this point, again further study is required.

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