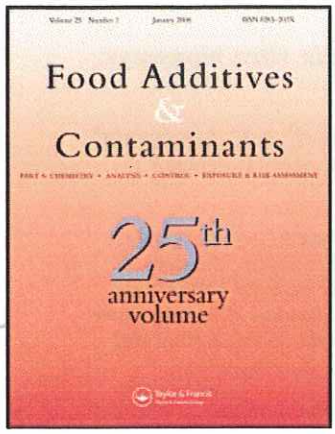

Food Additives & Contaminants: Part A
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713599661

Safety evaluation of food contact paper and board using chemical tests and in vitro bioassays: role of known and unknown substances

U. Honkalampi-Hämäläinen [a]; E. L. Bradley [b]; L. Castle [b]; I. Severin [c]; L. Dahbi [c]; O. Dahlman [d]; J. -C. Lhuguenot [c]; M. A. Andersson [e]; P. Hakulinen [f]; D. Hoornstra [e]; J. Mäki-Paakkanen [e]; M. Salkinoja-Salonen [e]; L. Turco [g]; A. Stammati [g]; F. Zucco [h]; A. Weber [i]; A. von Wright [a]
[a] Department of Biosciences, University of Kuopio, Kuopio, Finland [b] Central Science Laboratory, Food Safety and Quality, York, UK [c] Food Toxicology Laboratory, Université de Bourgogne, Dijon, France [d] Innventia AB, Stockholm, Sweden [e] Department of Applied Chemistry and Microbiology, University of Helsinki, Helsinki, Finland [f] The National Institute for Health and Welfare, Kuopio, Finland [g] Department of Environment and Primary Prevention, Istituto Superiore di Sanita, Rome, Italy [h] Consiglio Nazionale delle Richerche, Istituto Neurobiologia e Medicina Molecolare, Rome, Italy [i] KCL Science and Consulting, Espoo, Finland

First published on: 18 January 2010

To cite this Article Honkalampi-Hämäläinen, U., Bradley, E. L., Castle, L., Severin, I., Dahbi, L., Dahlman, O., Lhuguenot, J. -C., Andersson, M. A., Hakulinen, P., Hoornstra, D., Mäki-Paakkanen, J., Salkinoja-Salonen, M., Turco, L., Stammati, A., Zucco, F., Weber, A. and von Wright, A.(2010) 'Safety evaluation of food contact paper and board using chemical tests and in vitro bioassays: role of known and unknown substances', Food Additives & Contaminants: Part A, 27: 3, 406 — 415, First published on: 18 January 2010 (iFirst)
To link to this Article: DOI: 10.1080/19440040903401358
URL: http://dx.doi.org/10.1080/19440040903401358

Food Additives and Contaminants
Vol. 27, No. 3, March 2010, 406–415

Taylor & Francis
Taylor & Francis Group

Safety evaluation of food contact paper and board using chemical tests and *in vitro* bioassays: role of known and unknown substances

U. Honkalampi-Hämäläinen[a], E.L. Bradley[b], L. Castle[b], I. Severin[c], L. Dahbi[c], O. Dahlman[d], J.-C. Lhuguenot[c], M.A. Andersson[e], P. Hakulinen[f], D. Hoornstra[e], J. Mäki-Paakkanen[e], M. Salkinoja-Salonen[e], L. Turco[g], A. Stammati[g], F. Zucco[h], A. Weber[i] and A. von Wright[a]*

[a]*Department of Biosciences, University of Kuopio, Kuopio, Finland;* [b]*Central Science Laboratory, Food Safety and Quality, York, UK;* [c]*Food Toxicology Laboratory, Université de Bourgogne, Dijon, France;* [d]*Innventia AB, Stockholm, Sweden;* [e]*Department of Applied Chemistry and Microbiology, University of Helsinki, Helsinki, Finland;* [f]*The National Institute for Health and Welfare, Kuopio, Finland;* [g]*Department of Environment and Primary Prevention, Istituto Superiore di Sanita, Rome, Italy;* [h]*Consiglio Nazionale delle Richerche, Istituto Neurobiologia e Medicina Molecolare, Rome, Italy;* [i]*KCL Science and Consulting, Espoo, Finland*

(*Received 7 January 2009; final version received 8 October 2009*)

In vitro toxicological tests have been proposed as an approach to complement the chemical safety assessment of food contact materials, particularly those with a complex or unknown chemical composition such as paper and board. Among the concerns raised regarding the applicability of in vitro tests are the effects of interference of the extractables on the outcome of the cytotoxicity and genotoxicity tests applied and the role of known compounds present in chemically complex materials, such as paper and board, either as constituents or contaminants. To answer these questions, a series of experiments were performed to assess the role of natural substances (wood extracts, resin acids), some additives (diisopropylnaphthalene, phthalates, acrylamide, fluorescent whitening agents) and contaminants (2,4-diaminotoluene, benzo[*a*]pyrene) in the toxicological profile of paper and board. These substances were individually tested or used to spike actual paper and board extracts. The toxic concentrations of diisopropylnaphthalenes and phthalates were compared with those actually detected in paper and board extracts showing conspicuous toxicity. According to the results of the spiking experiments, the extracts did not affect the toxicity of tested chemicals nor was there any significant metabolic interference in the cases where two compounds were used in tests involving xenobiotic metabolism by the target cells. While the identified substances apparently have a role in the cytotoxicity of some of the project samples, their presence does not explain the total toxicological profile of the extracts. In conclusion, in vitro toxicological testing can have a role in the safety assessment of chemically complex materials in detecting potentially harmful activities not predictable by chemical analysis alone.

Keywords: bioassay; toxicology; cytotoxicity; food contact materials; mutagenic compounds; packaging

Introduction

Paper and board materials intended for food contact applications are chemically complex matrices. Owing to their long history of use, the manufacturing processes used and the fact that they are made mainly from naturally occurring raw materials, paper and board has a good reputation with the consumer. While there are either regulations or recommendations in some individual Member States of the European Union (EU), there are no specific EU-harmonised Directives or Regulations on paper and board. Rather, the general Framework Regulation No. (EC) 1935/2004 is invoked. The central provision applying to all food contact materials is as follows:

"Materials and articles must be manufactured in compliance with good manufacturing practice so that, under their normal or foreseeable conditions of use, they do not transfer their constituents into foodstuffs in quantities which could either endanger human health or bring about an unacceptable change in the composition of the foodstuffs or deterioration in the organoleptic characteristics thereof".

To demonstrate this is a challenge. Whereas chemical analysis is used to test plastics for the migration of known constituents, such as monomers and additives, into foods or food simulants, chemical analysis is less informative in the case of materials such as paper and board which can contain a large number of both known and unknown substances. Testing by bioassay of the global (total) extract or migrate from paper/board can complement chemical analysis by providing additional information. Toxicological screening tests

*Corresponding author. Email: atte.vonwright@uku.fi

ISSN 0265-203X print/ISSN 1464-5122 online

DOI: 10.1080/19440040903401358
http://www.informaworld.com

that could be applied to paper and board food contact materials have been reviewed recently (von Wright 2007).

The EU project BIOSAFEPAPER (QLK1-CT-2001-00930) was designed to address the safety aspects of food contact paper and board by in vitro toxicological screening or checking, whether sufficient amounts of substances would migrate to a selected food simulant to elicit a biological response in short-term bioassays in conditions mimicking the actual consumer exposure. The cytotoxicity and genotoxicity assays selected for this purpose have been described (Bradley et al. 2008).

The ethanol extract of two board samples, designated NSP4 (a non-food grade material) and NSP5 proved to be strongly cytotoxic and the former also mutagenic in the Ames test (Bradley et al. 2008). In chemical analysis, the majority of the identified extractables in NSP4 were diisopropylnaphthalene (DiPN) isomers, alkanes, different phthalates and resin acids; while, in NSP5, the most abundant identified compound was 1-methyl-2-pyrrolidinone (Table 1).

The purpose of the experiments described in this paper was to examine the role of the identified substances in the toxicity of the NSP4 extract. Additionally, the responses of the tests to either natural constituents of the raw materials for paper and board manufacture, substances used for special technical purposes (fluorescent whitening agents or FWAs) or different contaminants were examined. These substances were tested as such and they were also used to spike actual extracts of paper and board to ascertain the eventual interactions between them and other extractables present in the samples.

Table 1. Quantitatively the most important compounds identified in ethanol extracts of board samples NSP4 and NSP5 (Bradley et al. 2008).

	Concentration ($\mu g\,ml^{-1}$)	
Compound	NSP4	NSP5
Diisopropylnaphthalene isomers	218	37
C15–29 *n*-alkanes	154	116
Diisobutyl phthalate	120	65
Dibutyl phthalate	37	10
Di(2-ethylhexyl)phthalate	29	20
Other phthalates	5.5	3,4
1-Methyl-2-pyrrolidininone	not detected	290
Dehydroabietic acid and derivatives	40.9	24.5
Sum	604	565
Fraction of the total amount of extractables	80%	63%

Materials

Test substances

Spruce wood water extract

Spruce wood meal (5 g) was extracted by shaking with 100 ml water at 50°C for 4 h. The extract was filtered and stored refrigerated before use to prevent microbial growth. A portion was evaporated to dryness and this revealed that the extract contained ca. $7.0\,g\,l^{-1}$ of dry matter.

Spruce wood ethanol extract

Spruce wood meal (5 g) was extracted by shaking with 100 ml ethanol at room temperature for 4 h. The extract was filtered and stored refrigerated. It contained $1.2\,g\,l^{-1}$ dry material.

Other chemicals

The tetra-sulpho and disulpho-stilbene FWAs were from Bayer (supplied as $40\,g\,l^{-1}$ aqueous solutions by the paper industry partners in the project). 2,4-Diaminotoluene (98%), dibutylphthalate (DBP, 99+%) and di(2-ethylhexyl)phthalate (DEHP, 99+%) were from Aldrich (Gillingham, UK). Diisobutylphthalate (DiBP, Bisoflex, commercial plasticiser, ex. BP Chemicals Ltd., London UK, no purity given) was provided by FERA. Acrylamide (>99%) and benzo[*a*]pyrene (B[*a*]P, were from Sigma (Poole, UK). Diisopropylnaphthalenes (DiPNs, isomeric mixture, "reagent grade") was from Fisher (Loughborough, UK). Dehydroabietic acid was a technical grade sample available within STFI. Analysis by GC–MS revealed that it constituted 95% dehydroabietic acid, and three other minor resin acids made up the remaining 5%. For the cytotoxicity tests, the compounds were dissolved either in DMSO (phthalates, DiPNs,) or methanol (acrylamide, 2,4-diaminotoluene, and for the genotoxicity assays in DMSO.

Paper and board extracts for spiking experiments

A cold water extract from recycled test liner (BSP5), prepared according to EN 647 standard, and an ethanol extract from bleached kraft (BSP9), prepared according to pre-standardization method ENV 1186–15, were used in spiking experiments with acrylamide. The total content of migrants in the respective final extracts was $16.1\,mg\,l^{-1}$ (BSP5) and $46\,mg\,l^{-1}$ (BSP9). These extracts were selected for their low toxicity in preliminary cytotoxicity assays. The water extract of uncoated SBS (Solid Bleached Sulfate) NSP11, due to its high content of extractables ($472\,mg\,l^{-1}$), and the ethanol extract of NSP4, for its high cytotoxicity

(for details of these extracts, see Bradley et al. 2008), were selected for spiking with B[*a*]P and DiPNs.

Methods

Cytotoxicity assays

Tests with human larynx carcinoma cell line (HEp-2) and metabolically competent mouse hepatoma cell line (Hepa–1c1c7)

The tests were performed as described in detail by Severin et al. (2005) and Bradley et al. (2008) using either the total protein content (TPC) or the neutral red uptake (NRU) as endpoints; the former with Hepa–1c1c7 cells and the latter with HEp-2 cells.

RNA-synthesis inhibition test with HepG2 cells

In vitro RNA synthesis inhibition was measured by an automated test as a 30-min kinetic measurement of the uptake of tritiated uridine into the cellular macromolecules, as described by Severin et al. (2005).

Inhibition of boar spermatozoan motility

The extended boar semen, a commercial product obtained from an artificial insemination station, contained 27×10^6 spermatozoa ml^{-1} with 2–5 mM of extracellular potassium and 150–200 mM sodium. The extended semen was exposed to 1 vol% of the test substance or its dilutions for 1–4 days at room temperature, after which the inhibition of sperm motility was assessed (Andersson et al. 1998).

Genotoxicity assays

Ames test

The standard Ames test (Maron and Ames 1983), based on histidine auxotrophic *Salmonella typhimurium* strains TA98, TA100 and TA97, was performed on the following chemicals: acrylamide (10 µg–10 mg/plate); 2,4-diaminotoluene (2.5–2000 µg/plate); phthalate mixture (5 µg–500 µg/plate); DiPNs (5 µg–500 µg/plate); disulpho-stilbene (5 µg–500 µg/plate); tetrasulpho-stilbene (5 µg–500 µg/plate).

The S-9 preparation for metabolic activation system was either a commercial preparation (IFFA CREDO, L'Arbresle, France) or was obtained from phenobarbital/β-naphtoflavone-induced rat liver prepared by the National Laboratory Animal Center, University of Kuopio. Both microsomal preparations were found to perform equally with routine positive controls (data not shown).

Comet assay with HepG2 cells

The in vitro Single Cell Gel/Comet assay was performed as described by Bradley et al. (2008). Since HepG2 cells are metabolically competent, no metabolic activation was needed. Briefly, the HepG2 cells were grown in conditions identical to those applied for the RNA-synthesis inhibition test. For the test, six wells of a 96-well tissue culture plate per concentration were seeded with 5×10^4 cells in 0.2 ml of the culture medium. After 20 h of incubation, the cells were treated with the test substance for 20 h.

After the exposure, the cells mixed in 75 µl of low-melting point agarose were spread on slides pre-coated with normal melting point agarose. After cell lysis, electrophoresis was performed followed by staining of DNA and analysed. Olive tail moment (OTM, a measure of tail length × a measure of DNA in the tail) was used as the metric to characterise DNA damage in individual cells. Analysis was done using an automated image analysis system (Comet, version 4; Kinetic Imaging, UK). A total of 100 (50 per slide) individual comets were analysed per sample.

Results

Cytotoxicities of the tested chemicals or extracts

Results of the different cytotoxicity assays are summarized in Table 2. There was a general agreement between the acute cytotoxicity and the RNA-synthesis inhibition tests, with the exception of spruce wood extracts. Also, the outcome of the boar spermatozoan motility inhibition assay agreed with the traditional cytotoxicity assays.

Spruce wood extracts generally showed only marginal toxicity (mainly in HEp-2 cells). Of the different pure substances tested on the mammalian cells, the most toxic was 2,4-diaminotoluene followed by acrylamide, tetrasulpho-stilbene and disulpho-stilbene FWAs. The DiPNs mix was consistently more toxic than the phthalate mixture (it should be noted that, in this case, the results are expressed as microgram per millilitre and not as molar toxicities due to practical reasons). The hepatic cell lines had a different sensitivity to some of the test agents from that of the Hep-2 cell line. Compounds, such as 2,4-diaminotoluene and FWAs were more toxic to HepG2 and Hepa-1c1c7 cells than to HEp-2 cells, while the opposite was true with DiPNs and phthalates.

Tests on spiked paper and board samples

Acrylamide-spiked extracts

The effect of paper and board extracts on the cytotoxicity of acrylamide was tested in Hepa–1 cells

Table 2. Cytotoxic and genotoxic properties of wood-related substances and model contaminants.

		Cytotoxicity					Genotoxicity
				Immediate cytotoxicity			
Chemical or extract	Concentration range	Boar spermato-zoan motility inhibition	RNA-synthesis inhibition (HepG2 cells)	Hepa-1c1c7 cells	HEp-2 cells	Comet assay[a]	Ames test[b]
Dehydroabietic acid	0.25–2 mg ml^{-1}	NE	NE	NE	NE	Negative 0.1 mg ml^{-1}	Negative 800 g
Spruce wood (ethanol extract)	0.25–2%	NE	$EC_{50} \approx 1.3$ $EC_{40} \approx 1.0$	NE	NE	Negative	NT
Spruce wood (water extract)	1–90%	NE	$EC_{50} \approx 33$ $EC_{40} \approx 25$	NE	$EC_{50} \approx 20$	Negative	NT
Acrylamide	1–16 mM	5	$EC_{50} \approx 4$ $EC_{40} \approx 3.2$	$EC_{50} \approx 2.5$ $EC_{20} \approx 2$	$EC_{50} \approx 4$ $EC_{20} \approx 1$	Positive 1.5 mM	Negative 10 mg
2,4-Diamino-toluene	1–45 mM	1	$EC_{50} \approx 4.3$ $EC_{40} \approx 2.4$	$EC_{50} \approx 1$ $EC_{20} \approx 0.1$	$EC_{50} \approx 22$ $EC_{20} \approx 5$	Positive 1 mM	Positive 5 µg
Phthalate mixture	7–150 µg ml^{-1}	8	$EC_{50} \approx 99$ $EC_{40} \approx 83$	$EC_{50} \approx 13$ $EC_{20} \approx 9$	$EC_{50} \approx 2.5$ $EC_{20} \approx 2$	Negative 800 µg ml^{-1}	Negative 500 µg
Diisopropyl-naphthalene isomers(DiPNs)	2.5–74 µg ml^{-1}	2.5	$EC_{50} \approx 20$ $EC_{40} \approx 35$	$EC_{50} \approx 15$ $EC_{20} \approx 10$	$EC_{50} \approx 13$ $EC_{20} \approx 1.4$	Negative 80 µg ml^{-1}	Negative 500 µg
Disulpho-stilbene	0.1–12 µg ml^{-1}	0.2	$EC_{50} \approx 0.7$ $EC_{40} \approx 0.5$	$EC_{50} \approx 1.4$ $EC_{20} \approx 0.9$	$EC_{50} \approx 5.0$ $EC_{20} \approx 0.1$	Negative	Negative 500 µg
Tetrasulpho-stilbene	1–46 µg ml^{-1}	0.4	$EC_{50} \approx 5.6$ $EC_{40} \approx 3.7$	$EC_{50} \approx 5.5$ $EC_{20} \approx 2$	$EC_{50} \approx 19$ $EC_{20} \approx 1$	Negative	Negative 500 µg

Notes: [a]Concentration is the highest tested if different from the top concentration given in the second column.
[b]Concentrations used in Ames tests were the highest amount per plate not causing growth inhibition of the tester strains (see text).
NE, No or only marginal effects even at the highest tested concentration.
NT, not tested.

and in the RNA-synthesis inhibition test with HepG2 cells. Also, the inhibitory concentrations for boar spermatozoan motility were determined.

The spiking of paper and board extracts with acrylamide did not produce any major effect on the toxicity of the substance in most of the tests (Table 3). Some synergistic action could be seen in the boar spermatozoan motility inhibition assay with BSP9 ethanol extract. Generally, the differences probably reflect the normal fluctuation of biological test systems rather than actual different response.

The positive response of acrylamide in the comet assay was not affected by the extractables present in the water extract of BSP5 or ethanol extract of BSP9 (data not shown).

B[a]P-spiked extracts

As the NSP4 ethanol extract was cytotoxic in the RNA synthesis inhibition assay (Bradley et al. 2008), it was tested in the comet assay at a concentration which induces a 50% decrease in the RNA synthesis to avoid a false positive response in genotoxicity due to cell death by apoptosis or necrosis.

A dose-dependent increase in the genotoxic effect due to B[*a*]P was observed. The effect was not influenced by the solvent (NSP4 extract versus ethanol or NSP11 extract versus water). However, the effect of DiPNs was variable. With NSP4, DiPNs did not influence the results at the lowest concentration of B[*a*]P (1 µM), but a decrease in genotoxicity appeared in cells treated with DiPNs together with 5 or 10 µM B[*a*]P (Figure 1). With NSP11, a slight increase in genotoxicity appeared at 6.25 µM B[*a*]P when tested together with DiPNs, but there was no effect at lower B[*a*]P concentrations (Figure 2).

Discussion

Rationale for the selection of the control substances tested

Spruce wood extracts

Hot water and ethanol extracts of spruce wood were chosen to represent the complex mixtures of natural substances potentially present in paper and board and which might interfere with the outcome of the toxicity tests performed on paper and board samples. Among the most abundant substance classes identified by GC–MS were lignans (9%), resin acids (8%), fatty acids (1%) and various alkanols (3%) (data not shown).

Table 3. Toxicities of acrylamide-spiked paper and board extracts.

Solvent	EC_{50} of acrylamide[a]			Inhibitory concentration of acrylamide
	Cytotoxicity (NRU, 24 h) in HEp-2 cells	Cytotoxicity (TPC, 24 h or 72 h) in Hepa-1 cells	RNA-synthesis inhibition in HepG2 cells	Boar spermatozoan motility inhibition assay (72 h)
Water	6.4 mM	9.4 mM (24 h) 3.5 mM (72 h)	4.09 mM	Non-inhibitory at 1.6 mM
Water extract of BSP5	9.6 mM	6.0 mM (24 h) 3.1 mM (72 h)	3.98 mM	Non-inhibitory at 1.6 mM
Ethanol (1% in the final test)	6.4 mM	6.2 mM (24 h) 2.0 mM (72 h)	3.27 mM	20.1 mM
Ethanol extract of BSP9 (1% in the final test)	4.8 mM	6.5 mM (24 h) 2.3 mM (72 h)	3.98 mM	5.1 mM

Notes: [a]These represent a different set of tests than those reported in Table 2.

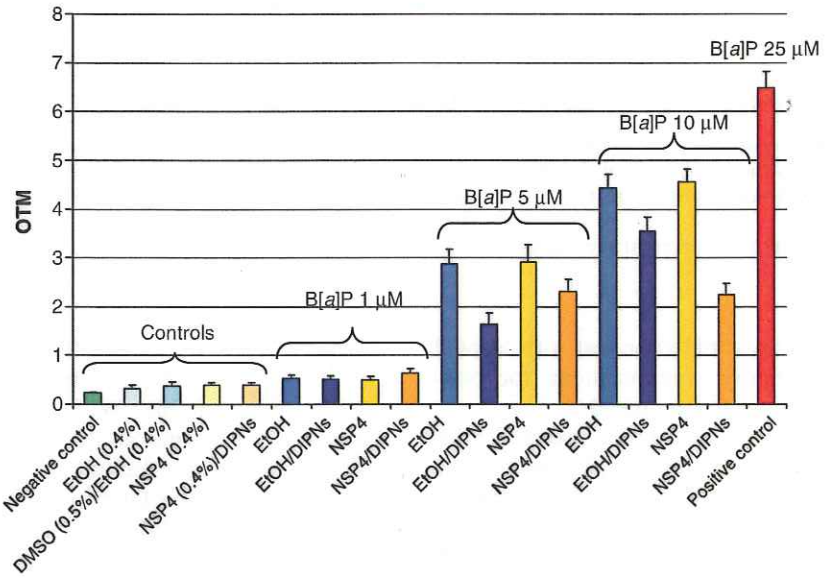

Figure 1. Interaction of DiPNs on the genotoxic effect of benzo[*a*]pyrene (B[*a*]P)-spiked ethanol extract of NSP4 in comet assay.

However, only about 20% of the organic material in the water extract could be identified by GC–MS.

Dehydroabietic acid

Dehydroabietic acid was chosen as representative of the natural resin acids which are normal constituents of wood. Natural resins are water-insoluble mixtures of substances, many of which have a hydroaromatic structure. Some resin acids are toxic to insects, moulds and fungi and protect the wood from attack. Depending on the paper-making process used, most resin acids are released and removed in the spent pulping-process liquor. In a small survey, total amounts of dehydroabietic acid and abietic acid were up to 1 mg g^{-1} (0.1%) in the samples tested (Ozaki et al. 2005). The same authors reported that dehydroabietic acid and abietic acid were positive in the in vitro Rec assay.

Fluorescent whitening agents (FWAs)

Di- and tetra-suphostilbenes were used as examples of FWAs due to their widespread use to enhance the brightness of paper and board; FWAs are also know as optical brighteners. Approximately 80% of FWAs used in paper and board manufacture are based on stilbene derivatives. They are quite planar/linear molecules with extensive delocalised π electron systems and one or more sulphonic acid groups (Roberts 1991)

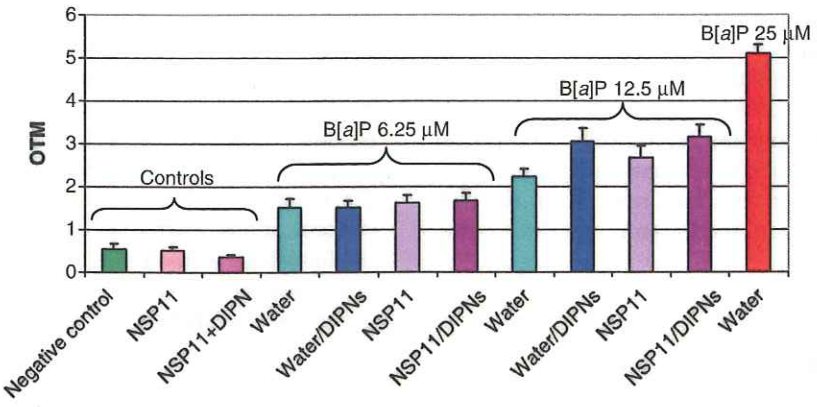

Figure 2. Absence of interaction of DiPNs on the genotoxic effect of benzo[*a*]pyrene (B[*a*]P)-spiked water extract of NSP11 in comet assay.

The tetra-sulpho stilbenes are by far the most commonly found, whereas the hexa-sulphos are rarely if ever used because they are too water soluble and so are not retained on the paper fibres to exert the effect intended. For these reasons, a tetra-sulpho and a di-sulpho-stilbene were selected as test substances. The substances selected are on the positive list of FWAs accepted in French and Italian regulations, and also are on the list of substances of the Council of Europe Resolution on Paper and Board (COE 2002).

Diisopropylnaphthalene

Diisopropylnaphthalene (DiPN) was selected because it can be considered as a typical process contaminant present in paper and board. Diisopropylnaphthalenes are isomeric mixtures in which the 2,6-form is the dominant di-alkylated isomers. DiPN was first detected in food packaging samples in 1994 (Sturaro et al. 1994) and originates mainly from the presence of carbonless copy papers in paper recovered for recycling. Levels in paper/board can be in the range 2–60 mg kg^{-1} (Bebiolka et al. 1997; MAFF 1999; Zhang et al. 2008). DiPN has a boiling point of 290–300°C and is virtually insoluble in water but is freely soluble in fats and organic solvents such as ethanol (RKS 1998). Although DiPN itself is relatively non-toxic, DiPN was included to test for any metabolic interference, e.g. saturation effects with competitive inhibition preventing the potent activity of B[*a*]P being displayed.

Acrylamide

Acrylamide is the starting substance for very high molecular weight polyacrylamides used in papermaking as wet-strength agents and as retention aids. Attention focussed on acrylamide in 2002 when it was discovered that it could form from normal components when many foods are cooked at high temperature. The polyacrylamides used in papermaking have either extremely low or usually even not-detectable residues of unreacted acrylamide.

Consequently, acrylamide is generally not detectable in paper/board (FSA 2002) and worst-case migration calculations are all well below the migration limit set for plastics of "not detectable", less than 10 µg kg^{-1} food.

Phthalates

Like DiPN, phthalates were selected because they can be considered as a typical process contaminants present in paper and board. Phthalates are a family of chemical with different toxicological properties. They are ubiquitous environmental contaminants and they may find their way into paper/board, particularly from their use in printing inks and glues (adhesives) that are not completely removed from recovered paper used in recycling. By far the most common phthalates encountered are DiBP, DBP and DEHP. They typically occur in a ratio that is rather constant at ca. 4 : 1 : 1.

2,4-Diaminotoluene and benzo[a]pyrene

These were chosen to represent typical widely occurring environmental pollutants of two classes, aromatic amines and polycyclic aromatic hydrocarbons, respectively. They are not expected to contaminate paper/board samples, but their toxic properties have been investigated thoroughly and so they were included as positive-control substances of interest.

Responses of the different cytotoxicity assays to the test substances

In general, there was good agreement between the results of the different mammalian cytotoxicity tests,

including the boar spermatozoan motility inhibition assay. Spruce wood extracts showed some toxicity in the RNA-synthesis inhibition assay, but were generally negative in other cytotoxicity tests, with the exception of the water extract and the HEp-2 cells. The special sensitivity of this cell line in comparison to the hepatic cell lines used in other tests may result from the lack of active detoxification mechanisms present in the liver cells.

Toxicological profile of the test compounds

Wood-associated substances

Both water and ethanol extracts of spruce wood evoked some toxic responses, mainly in the RNA-synthesis inhibition assay measuring sublethal effects; the water extract also showing some cytotoxicity in HEp-2 cells (Table 2). In contrast, dehydroabietic acid, which was the most abundant identified wood-associated compound in the actual sample, was consistently non-toxic at the tested concentrations. The water extracts of paper and board tested by us have been consistently non-toxic, and ethanol extracts also showed a wide variation in toxicity, some showing only marginal activity (Bradley et al. 2008). Thus, while there are apparently bioactive compounds present in wood, their eventual presence in paper and board does not interfere with the toxicological testing.

Cytotoxic profile of DiPNs and phthalates

DiPNs and phthalates were among the clearly cytotoxic substances identified in this study. In particular, the toxicity of phthalates was more pronounced in HEp-2 cells lacking an efficient xenobiotic metabolism compared to metabolically competent hepatic cell lines. This indicates some metabolic detoxification of these types of compounds.

DiPNs have been considered as a safe alternative for polychlorinated biphenyls in carbonless copy papers due to its reported low toxicity, which apparently results from the metabolic oxidation of the isopropyl side chain rather than the aromatic ring (Höke and Zellerhof 1998). No recent reports of either cytotoxicity or genotoxicity of DiPNs have been published and while, according to the results reported here, there are no indications of genotoxic concerns, the migration of amounts approaching the cytotoxic levels into foods or simulants remains a possibility.

Phthalates have attracted attention as endocrine disruptors and reproductive and developmental toxicants. Their cytotoxic properties have not been systematically studied. While they have been found non-mutagenic in the bacterial test systems (Yoshikawa et al. 1983, Zeiger et al. 1985), indications of DNA damage in human mucosal cells and lymphocytes have, however, been detected (Kleinsasser et al. 2000, 2001).

The human tolerable daily intakes (TDIs) proposed by different scientific bodies range from 0.020 mg kg^{-1} bw day^{-1} (infants) to 0.050 mg kg^{-1} bw day^{-1} for the di-(2-ethyl-hexyl) phthalate, which is generally regarded as the most toxic (Heudorf et al. 2007). Although the concentrations migrating from paper and board to the foodstuffs would not reach the TDIs proposed, the possibility of amounts approaching cytotoxic levels cannot be excluded.

Cytotoxic activity of FWAs and 2,4-diaminotoluene

Both FWAs and especially 2,4-diaminotoluene displayed a more marked toxicity in hepatic cells than in the HEp-2 cell line, this being an example of metabolic activation. This (together with the opposite results obtained with DiPNS and phthalates) illustrates the need to include cell lines with different metabolic capacities in toxicological test batteries to get a complete understanding of the bioactivities of the substances tested.

The FWAs used in the studies are stilbene derivatives. Natural stilbenes have antimicrobial and also cytotoxic effects (Välimaa et al. 2007), but are not known to be particularly toxic to humans; indeed, beneficial effects (antitumorigenic and antiartherosclerotic activities, protection against type II diabetes) have been associated with resveratrol, a natural stilbene (Pace-Aciak et al. 1995; Jang et al. 1997; Lagogue et al. 2006). In the studies reported here, both disulpho- and tetrasulpho-stilbenes were cytotoxic in all the test systems.

Tests for any bleeding of FWAs are frequently conducted by industry to ensure quality. Tests can be either optical (i.e. visual) or chemical (e.g. LC–FLD). Unlike many substances which might migrate from FCMs into food, FWAs are more soluble in water than fat. Migration of the water-soluble FWAs would be expected to be more extensive into moist foods rather than fatty foods. European Standard EN 648:2003 is concerned with the determination of the fastness of fluorescent-whitened paper and board (EN 648:2003). The paper/board sample is brought into contact with a glass-fibre paper which has been saturated with the test fluid (water, 3% acetic acid, 5 g l^{-1} sodium carbonate or olive oil). Transfer is assessed using reference discs made using an FWA standard at 0, 4.2, 11.3, 44 and 177 µg dm^{-2}. These represent grades 1 to 5. Grade 1 is descried as good colour fastness, grade 5 is described as poor colour fastness. The test conditions with a liquid simulant are rather severe.

Nevertheless, the middle calibration standard, Grade 3, is equal to a transfer of 11.3 µg dm^{-2}, which is just 68 ppb for the standard EU cube of 1 kg food in direct contact with 6 dm^{-2} of packaging. When FWAs

are surveyed (MAFF 1995), the conclusions are that, even assuming total mass transfer, there are no safety concerns. Therefore, the possibility of a consumer being accidentally exposed to cytotoxic concentrations of these substances, even in a worst case scenario assuming total migration to fatty foodstuff or drink, can be discounted.

2,4-Diaminotoluene was used as an example of primary aromatic amines (PAAs) that can be environmental pollutants. These chemicals are not expected to contaminate primary paper and board production but they can arise as contaminants from the use of certain aromatic isocyanates in adhesives or as impurities in some azo-dyes. For this reason, food contact materials are often tested for the release of PAAs using a colorimetric test with a requirement that PAAs should be not detectable at $10\,\mu g\,kg^{-1}$ food or food simulant. The value of $10\,\mu g\,kg^{-1}$, as the limit of the detection of the method, is the established limit for plastics because it has been decided that this type of substance should not migrate at all. The value is not linked to nor derived from any quantitative measure of toxic potency of PAAs. This value of $10\,\mu g\,kg^{-1}$ is more than a thousand times less than the toxic doses of 0.1 mM observed here in the most sensitive cell lines.

Experiences from the spiked samples

Acrylamide-spiked extracts

The purpose of these experiments was to check the effects of background material in the extracts on the cytotoxicity assays – acrylamide serving as a representative test substance. As there was no difference in the cytotoxic response between acrylamide dissolved either in water or ethanol or in the corresponding extracts of paper and board, the paper and board extractives apparently did not interfere with the assays. This conclusion should, however, be made with the reservation that the toxic concentrations are considerably higher than those that could be expected to occur in paper and board.

B[a]P-spiked extracts

The experiments were performed to find out the possible interference of two toxicants in the case when the toxic effects require metabolic transformation by the target cells. For that purpose, the known genotoxic substance B[*a*]P was tested at various concentrations in the Comet assay with the hepatic cell line HepG2 in the presence of DiPNs. The observed effects were minor (Figures 1 and 2) and could be considered as background variation inherent to the test. B[*a*]P was clearly genotoxic, irrespective of the extract into which it was dissolved or of the presence of DiPNs. Thus, the system appears to be robust, tolerating a considerable amount of metabolic interference.

Role of the identified substances in the toxicities of the NSP4 and NSP5 extracts

On the basis of the results reported here, the most cytotoxic substances present in significant amounts in the 50-fold concentrated NSP4 extracts would be DiPNs ($218\,\mu g\,ml^{-1}$) and phthalates ($192\,\mu g\,ml^{-1}$). At the clearly cytotoxic concentrations of the extract (0.5%; Bradley et al. 2008), these substances would have been present at quantities of around 1.09 and $0.96\,\mu g\,ml^{-1}$. When compared to the data presented in Table 3, these concentrations, being close to the EC_{20} values of some of the test systems, probably contribute to the cytotoxicity of NSP4. However, their amounts in the toxic doses of concentrated NSP5 extract are 0.19 and $0.45\,\mu g\,ml^{-1}$, respectively (Bradley et al. 2008) and neither of them alone would suffice to give a very potent response in the RNA-synthesis inhibition assays characteristic of NSP5.

No actual cytotoxicity studies on 1-methyl-2-pyrrolidinone, the most abundant identified compound of NSP5, has apparently been done, However, since this compound has been used to stimulate the differentiation of cultured kidney cells at concentrations of 25 mM (or $2475\,\mu g\,ml^{-1}$) (Lever 1979), it is unlikely to cause cytotoxicity at the concentrations reported here.

Dehydroabietic acid proved to be totally non-cytotoxic at the tested concentrations and, therefore, it or its derivatives probably do not have a role in the cytotoxicity of the extract.

While the combined effects of DiPNs and phthalates undoubtedly contribute to the cytotoxicity of the NSP extracts, the nature of the genotoxic principle in NSP4, given the absence of genotoxicity of either phthalates or DiPNs, remains unsolved. There are apparently no public genotoxicity reports on DiPNs, while the non-mutagenicity of phthalates in bacterial systems has been confirmed in several studies, as pointed out above. The other substances present in NSP4 are present in small quantities and are either tested non-mutagens (enzophenone, bisphenol A; Schweikl et al. 1998; Chhabra 2000) or their genotoxicity profile is unknown.

The difficulty in identifying compounds responsible for the observed toxicity of extracts of food contact materials is universal. In a recent report (Simat 2008), the Neutral Red Uptake test (NRU), which measures cytotoxicity as an inhibition of the uptake of a red dye in a cell culture of Hep G2 cells, was used along with in-depth chemical analysis for a comprehensive evaluation of the total migrate. This merits some detailed discussion because they were not testing paper/board, as done here, but can coatings and complex plastic laminates.

When testing the total migrate obtained from an epoxyanhydride can coating using 95% ethanol for 4 h

at 60°C, only about 0.5% of the inhibition could be attributed to regulated substances such as bisphenol A, bisphenol A diglycidyl ether and its hydrolysis product. Further chemical analysis revealed a major contribution (a further 18%) by cyclo-di-BADGE. There was no inhibition by the fraction with MW above 1000. This means that 80% of the NRT inhibition effect was caused by substances that remained unidentified.

A similar picture was revealed for the total migrate prepared using 95% ethanol for 4 h at 60°C on a plastic laminate. The laminate structure was complex: polyethylene/adhesive/polyethyleneterephthalate/aluminium (vacuum deposited)/ink/nitrocellulose lacquer. Chemical analysis identified 12 substances and, when these were tested in the NRT, eight were inactive and four accounted for 33% of the inhibition seen for the total migrate. When these four substances were tested in combination (not individually), there was a +40% enhancement; so that, in combination, they accounted for about 46% of the total inhibition seen. This still left more than 50% of the cytotoxicity caused by unidentified substances. As with the example of the can coating, this large missing fraction emphasises the need to use bioassays to help pinpoint and then identify any potentially hazardous substances.

Simat (2008) commented that some regulated substances show no response in the NRU assay and, for some substances, the NR uptake has insufficient sensitivity to test at the specific migration limit (SML). There are several reasons for this.

First, the NRU reveals only one mechanism of toxicity. The mechanistic principle of the test is that is substances which damage the lysosomal membrane then inhibit the uptake of NR. In reality, there are many mechanisms of toxicity and this reinforces the need for a battery of tests, as recommended here. Additionally, the sensitivity of cytotoxicity assays depends also of the cell line used, as pointed out above. Ideally, both hepatic cell lines capable of inactivating and, in some cases, activating xenobiotics and metabolically less competent cells (like Hep-2 cells used here) should be used in the testing programme.

Second, a regulated substance often has an SML based not on any toxicity seen but on the extent of the data package submitted (Barlow 2009). For the most common SML restrictions of 50 ppb and 5 ppm, there would have been no toxicity observed in the tests evaluated by EFSA and the SCF before them.

Finally, this intrinsic lack of sensitivity was compounded by a low tolerance of the NRU to the carrier solvent used (DMSO, dimethylsulfoxide) and only up to 1% could be added to the cell culture. DMSO is a polar, water-miscible solvent that has limited capacity to redissolve the total migrate obtained using a large volume of hot ethanol. Also, the total migrate from a can coating and from a plastic laminate will be rather non-polar in character and not well suited for DMSO. Solubility was less of a limiting factor for testing paper/board and the total migrate could, for example, be concentrated 10-fold and applied to the bioassays without problems (Bradley et al. 2008, 2009).

Conclusions

Cytotoxicity and genotoxicity assays can be used to assess the consumer safety of paper and board. While the toxic substances can, in some cases, be tentatively retrospectively identified, the large number of substances that can be present in the extracts and their eventual interactions make the pinpointing of cyto- or genotoxic activities to individual substances difficult or impossible. The sensitivities of the different assays are variable and also the tolerance of the biological test systems to solvents used in the extraction is typically low. This necessitates the use of a combination of different tests, including the use of cell lines with variable metabolic capacities, and often concentration steps for the samples.

Acknowledgements

This work was funded by the European Union under contract QLK1-2001-00930 "BIOSAFEPAPER – Application of bioassays for safety assessment of paper and board for food contact" and by a consortium of 16 paper and board making companies. The findings and the conclusions in this paper are the responsibility of the authors alone and they should not be taken to represent the opinion of the European Commission or any paper and board making companies.

References

Andersson MA, Mikkola R, Helin J, Andersson MC, Salkinoja-Salonen M. 1998. A novel sensitive bioassay for detection of *Bacillus cereus* emetic toxin and related depsipeptide ionophores. Appl Environ Microbiol. 64:1338–1343.

Barlow SM. 2009. Risk assessment of food contact materials: Past experience and future challenges. Proceedings of the 4th International Symposium on Food Packaging, Prague, November 2008. Food Addit Contam. 26:1526–1533.

Bebiolka H, Dunkel K. 1997. Übergang von di-iso-propylnaphthalin aus karton-verpackungen auf lebensmittel (Passage of diisopropylnaphthalene from cardboard packing to food). Lebensmittelchemie 51:53–61.

Bradley EL, Honkalampi-Hämäläinen U, Weber A, Andersson MA, Bertaud F, Castle L, Dahlman O, Hakulinen P, Hoornstra D, Lhuguenot J-C, et al. 2008. The BIOSAFEPAPER project for in vitro toxicity assessments: Preparation, detailed chemical characterisation and testing of extracts from paper and board samples. Food Chem Toxicol. 46:2498–2509.

Bradley EL, Honkalampi-Hämäläinen U, Andersson MA, Bertaud F, Castle L, Dahlman O, Hakulinen P, Hoornstra D, Lhuguenot J-C, Mäki-Paakkanen J,

et al. 2009. Test procedures for obtaining representative extracts suitable for reliable in vitro toxicity assessment of paper and board intended for food contact. Food Addit Contam. In press.

Chhabra RS. 2000. NTP technical report on the toxicity studies of benzophenone (CAS No. 119-61-9). Administered in feed to F344/N rats and B6C3F mice. Toxicity Report Series 61:1–53.

COE 2002. Council of Europe Resolution A.P 2002.1 on paper and board materials and articles intended to come into contact with foodstuffs.

EN 648:2003. Paper and board intended to come into contact with foodstuffs. Determination of the fastness of fluorescent whitened paper and board. European Standard EN 648:2003.

FSA 2002. Paper and board packaging: not likely to be a source of acrylamide in food. Food Survey Information Sheet 27/02. London: Food Standards Agency.

Heudorf U, Mersch-Sunderman V, Angerer J. 2007. Phthalates: Toxicology and exposure. Int J Hyg Environ Health 210:623–634.

Höke H, Zellerhoff R. 1998. Metabolism and toxicity of diisopropylnahthalene as compared to naphthalene and monoalkyl naphthalenes: a minireview. Toxicology 126:1–7.

Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CWW, Fong HHS, Farnsworth NR, Kinghorn AD, Mehta RG, et al. 1997. Cancer chemopreventive action of resveratrol, a natural product derived from grapes. Science 275:218–220.

Kleinsasser NH, Kastenbauer ER, Weissacher H, Muenzenrieder RK, Harréus UA. 2000. Phthalates demonstrate genotoxicity on human mucosa of the upper aerodigestive tract. Environ Mol Mutagen. 35:9–12.

Kleinsassner NH, Wallner BC, Kastenbauer ER, Weissacher H, Harréus UA. 2001. Genotoxicity of di-butyl-phthalate and di-isobutyl-phthalate in human lymphocytes and mucosal cells. Teratogen Carcinogen Mutagen. 21:189–196.

Lagouge M, Argmann C, Gerhart-Hines. Z, Meziane H, Lerin C, Daussin F, Messadeq N, Milne J, Lambert P, Elliott P, et al. 2006. Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1α. Cell 127:1–14.

Lever JE. 1979. Inducers of mammalian cell differentiation stimulate dome formation in a differentiated kidney epithelial cell line (MDCK). Proc Natl Acad Sci USA 76:1323–1327.

MAFF 1995. Fluorescent whitening agents. Food Surveillance Information Sheet 47. London: Ministry of Agriculture Fisheries and Food.

MAFF 1999. Diisopropylnaphthalenes in food packaging made from recycled paper and board. Food Surveillance Information Sheet 169. London: Ministry of Agriculture Fisheries and Food.

Maron DM, Ames BN. 1983. Revised methods for the Salmonella mutagenicity test. Mutat Res. 113:173–215.

Ozaki A, Yamaguchi Y, Fujita T, Kuroda K, Endo G. 2005. Safety assessment of paper and board food packaging: Chemical analysis and genotoxicity of possible contaminants in packaging? Food Addit Contam. 22:1053–1060.

Pace-Asciak CR, Hahn S, Diamandis EP, Soleas G, Goldberg DM. 1995. The red wine phenolics *trans*-resveratrol and quercetin block human platelet aggregation and eicosanoid synthesis: implication for protection against coronary heart disease. Clin Chim Acta 235:207–219.

RKS 1998. DIPN (diisopropylnaphthalene) technical information (data sheet) July 1998. RKS GmbH, Duisburg, DE.

Roberts JC (editor). 1991. Paper Chemistry. Blackie, Glasgow. ISBN 0-2 16-92909-1.

Schweikl H, Schmalz G, Rackenbrandt K. 1998. The mutagenic activity of unpolymerized resin monomers in *Salmonella typhimurium* and V79 cells. Mutat Res. 415:119–130.

Severin I, Dahbi L, Lhuguenot J-C, Andersson MA, Hoornstra D, Salkinoja-Salonen M, Turco L, Zucco F, Stammati A, Dahlman O, et al. 2005. Safety assessment of food-contact paper and board using a battery of short-term toxicity tests: European Union BIOSAFEPAPER project. Food Addit Contam. 22:1032–1041.

Simat JT. 2008. Use of biotests in the analysis of migrants from coatings and plastic laminates. Paper presented at the 4th International Symposium on Food Packaging. November 19–21, Prague (Czech Republic).

Sturaro A, Parvoli G, Rella R, Bardati S, Doretti L. 1994. Food contamination by diisopropylnaphthalenes from cardboard packages. Int J Food Sci Technol. 29:593–603.

Välimaa A-L, Honkalampi-Hämäläinen U, Pietarinen S, Willför S, Holmbom B, von Wright A. 2007. Antimicrobial and cytotoxic knotwood extracts and related pure compounds and their effects on food-associated microorganisms. Int J Food Microbiol. 115:235–243.

von Wright A. 2007. Safety assessment of paper and board used in food packaging. In: Barnes KA, Sinclair R, Watson D, editors. Chemical migration and food contact materials. Cambridge, UK: Woodhead Publishing. p. 333–345, ISBN–13: 978–1-84569-029-8.

Yoshikawa K, Tanaka A, Yamaha T, Kurata H. 1983. Mutagenicity study of nine monoalkyl phthalates and a dialkylpthalate using *Salmonella typhimurium* and *Escherichia coli*. Food Chem Toxicol. 21:221–223.

Zeiger E, Haworth S, Mortelmans K, Speck W. 1985. Mutagenicity testing of di(2-ethylhexyl)phthalate and related chemicals in Salmonella. Environ Mutagen. 7:213–232.

Zhang K, Noonan GO, Begley TH. 2008. Determination of 2,6-diisopropylnaphthalene (DiPN) and *n*-dibutylphthalate (DBP) in food and paper packaging materials from US marketplaces. Food Addit Contam A 25:1416–1423.