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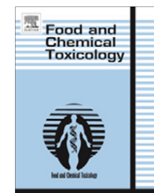

The BIOSAFEPAPER project for *in vitro* toxicity assessments: Preparation, detailed chemical characterisation and testing of extracts from paper and board samples

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ABSTRACT

Nineteen food contact papers and boards and one non-food contact board were extracted following test protocols developed within European Union funded project BIOSAFEPAPER. The extraction media were either hot or cold water, 95% ethanol or Tenax, according to the end use of the sample. The extractable dry matter content of the samples varied from 1200 to 11,800 mg/kg (0.8–35.5 mg/dm^2). According to GC–MS the main substances extracted into water were pulp-derived natural products such as fatty acids, resin acids, natural wood sterols and alkanols. Substances extracted into ethanol particularly, were diisopropylnaphthalenes, alkanes and phthalic acid esters. The non-food contact board showed the greatest number and highest concentrations of GC–MS detectable compounds. The extracts were subjected to a battery of *in vitro* toxicity tests measuring both acute and sublethal cytotoxicity and genotoxic effects. None of the water or Tenax extracts was positive in cytotoxicity or genotoxicity assays. The ethanol extract of the non-food contact board gave a positive response in the genotoxicity assays, and all four ethanol extracts gave positive response(s) in the cytotoxicity assays to some extent. These responses could not be pinpointed to any specific compound, although there appeared a correlation between the total amount of extractables and toxicity.

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1. Introduction

Food contact paper and board represent chemically complex materials with special challenges regarding their safety evaluation. The total amounts of extractables in different model systems can be high and the number of compounds considerable, including a large fraction of unknowns. Consequently, short-term bioassays have been proposed as an approach for safety assessment (von Wright, 2004), and to some extent they already have been used to detect various bioactivities present in fibre-based packaging materials.

Fauris et al. (1998) made a systematic survey on 6 paper and 15 board samples from different European countries using the RNA synthesis inhibition in exposed human HeLa cells as the toxicological endpoint. The samples represented both recycled and virgin fibres. CEN standard procedures were used in the analysis of water soluble matter and in the preparation of water extracts. According

Abbreviations: BSTFA, *N,O*-bis(trimethylsilyl)trifluoro-acetamide; CEN, Comité Européen Normalisation; Da, Daltons; DIPN, diisopropyl naphthalene; DMC, dry matter content; DNA, deoxyribonucleic acid; EU, European Union; GC–MS, gas chromatography–mass spectrometry; LMWF, low molecular weight fraction; HMWF, high molecular weight fraction; NRU, neutral red uptake; NSP, nominated sample for the project; OTM, olive tail movement; P/B, paper/board; RNA, ribonucleic acid; TPC, total protein content; UF, ultra-filtration

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to the results the cytotoxicity of the samples ranged from very high (RNA synthesis rate 17% of the control) to non-toxic (RNA synthesis rate 94%). The same range of toxicities was found for extracts of both recycled products and virgin fibres. Among the latter, the toxic samples represented mechanical pulps. The toxicities of the samples did not correlate with any individual analysed chemical component. Instead, there was a correlation between the toxicity and the numbers of peaks in the gas chromatogram.

Binderup et al. (2002) evaluated three categories of recycled fibre-based food contact papers in a test battery consisting of a cytotoxicity test on human skin fibroblasts, Ames test for genotoxicity, recombinant yeast test for estrogenic activity and CALUX-test for the detection of dioxin-like activity. The recycled papers were compared to virgin fibre. The samples were extracted both with 99% ethanol and water. The extracts were monitored for extractable substances and also subjected for microbiological analyses. Ethanol extracts showed more toxicity than water extracts and also contained higher amounts of material in the chemical analysis. The recycled products were consistently more toxic than the virgin fibre. None of the extracts gave a positive effect in the Ames test, and all were too cytotoxic to the recombinant yeast cell line to produce meaningful results. Signs of dioxin-like activity were detected in all ethanol extracts and with some water extracts.

Some estrogenic activity in a hormone responding cell line has subsequently been indicated in paper and cardboard extracts (Lopez-Espinosa et al., 2007), the authors suggesting a link between the hormone-like activity and levels of phthalates and bisphenol A in the samples.

Ozaki et al. (2004) studied both the chemical composition and genotoxicity of ethanol extracts of altogether 28 different food contact papers representing both virgin and recycled materials. Altogether 20 different contaminants were chemically analysed from the extracts, including, among others, Michler's ketone, and related benzophenone derivatives, hydroxyphenylpropane compounds, chlorophenols and other chlorinated aromatics. The genotoxicity test battery included a bacterial *rec*-assay (a differential killing assay using DNA-repair-proficient and repair-deficient *Bacillus subtilis* strains) and the Comet assay. Of the 12 extracts of recycled products nine were positive in the *rec*-assay, whereas only three of the 16 extracts of virgin materials showed genotoxic activity in this test. Eight extracts positive in the *rec*-assay were also subjected to the Comet assay, in which six proved to be positive. Significantly, three of the extracts that were positive in both assays were from virgin material. The levels of the contaminants identified in the extracts were too low to explain the positive toxicological responses. In subsequent work (Ozaki et al., 2005) the authors

Table 1
Sample description and selected extraction/migration test conditions for the 20 representative paper and board samples studied

Code	Description	Grammage (g/m^2)	Recycled content (%)	Intended food use	Time / temperature conditions of intended use	Simulant used and the ratio (g sample/l simulant or g Tenax/dm^2 sample)	Time/temperature conditions of testing
NSP1	Solid board	665	100	Dry	1 week at 4 °C	Tenax (1 g/dm^2)	24 h at room temperature
NSP2	Board GD2 (WLC)	300	84	Dry	Months at ambient temperature	Tenax (1 g/dm^2)	5 days at 50 °C
NSP3	Board GD2 high newsprint	300	100	Dry	Months at ambient temperature	Tenax (1 g/dm^2)	5 days at 50 °C
NSP4	Board GD3 (WLC)	300	100	Non-food grade	Not relevant non-food grade	95% Ethanol (100 g/l), water (40 g/l), Tenax (1 g/dm^2)	24 h at room temperature 24 h at 20 °C, 5 days at 50 °C
NSP5	Board GD grease-resistance treated	500	90	Fat	Months at ambient temperature	95% Ethanol (100 g/l)	24 h at room temperature
NSP6	Board GD water resistance treated	300	98	Wet	Minutes at ambient[a]	Water (40 g/l)	24 h at 20 °C
NSP7	Folding boxboard wet strengthened	285	0	Wet	1 week at 8 °C	Water (40 g/l)	24 h at 20 °C
NSP8	White top kraft liner	140	0	Fat	Minutes at hot[a]	95% Ethanol (100 g/l)	24 h at RT
NSP9	Unbleached liner recycled, surface sized	170	100	Dry	Weeks at ambient temperature	Tenax (1 g/dm^2)	5 days at 50 °C
NSP10	Bleached liner recycled	140	100	Dry	A few weeks at ambient temperature	Tenax (1 g/dm^2)	10 days at 20 °C
NSP11	Uncoated SBS	210	0	Wet	Minutes hot	Water (40 g/l)	24 h at 80 °C
NSP12	Recycled fluting dry food	105	100	Dry	Months at ambient temperature (indirect contact)	Tenax (1 g/dm^2)	5 days at 50 °C
NSP13	Corrugated board (virgin), pigment coated	666	0 (inner liner is 20% recycled)	Fat	Months at frozen[a]	95% Ethanol (100 g/l)	24 h at room temperature
NSP14	Corrugated board recycled	500	100	Dry	Up to 4 weeks frozen[a]	Tenax (1 g/dm^2)	10 days at 20 °C
NSP15	Unbleached sulphate kraft paper virgin	70	0	Dry	Months at ambient temperature	Tenax (1 g/dm^2)	5 days at 50 °C
NSP16	Paper based on deinked fibre	40	100	Dry	1 day (maximum) at ambient temperature	Tenax (1 g/dm^2)	24 h at room temperature
NSP17	Folding boxboard; pigment-coated	255	0	Dry	Months at ambient temperature[a]	Tenax (1 g/dm^2)	5 days at 50 °C
NSP18	Solid bleached board, pigment-coated	240	0	Wet	Months frozen	Water (40 g/l)	24 h at 20 °C
NSP19	CNB	300	0	Wet	Months at all temperatures	Water (40 g/l)	24 h at 20 °C
NSP20	Hard sized paper	30	80	Wet	1 day (maximum) at ambient temperature	Water (40 g/l)	24 h at 20 °C

Note: Nearly all producers reported internal sizing treatments.
[a] Indicates other uses or temperature also given.

indicated a role for dehydroabietic acid and abietic acid in the positive response seen in the *rec*-assay.

Thus, although relatively few studies on the *in vitro* toxicity of paper and board have been published, the reported results suggest that different short-term tests have the potential to indicate whether bioactive components leach out from the materials in test conditions mimicking the actual use. The EU 5th Framework project BIOSAFEPAPER ran from 2002 to 2006 and aimed to develop, validate and intercalibrate a short-term test battery for safety assessment of paper and board intended for food contact (Severin et al., 2005). This paper describes the phase of research conducted to prepare and test extracts of representative samples of paper and board materials intended to come into contact with foods. It describes the chemical analysis of these extracts, the toxicological findings using the test battery and the conclusions drawn from this work.

2. Materials and methods

2.1. Description of samples

Twenty samples of paper and board were selected by the industrial platform members of the BIOSAFEPAPER project and were submitted blind for extraction followed by chemical analysis and bioassay analysis of the extracts. The samples were coded NSP1 to 20. The materials were chosen as representative of the range of food contact applications in which paper and board packaging materials may be employed. One of the 20 samples, coded NSP4, was subsequently revealed to be a board not intended for food contact and it had been included for comparison purposes. The information provided with the samples is shown in the first six columns of Table 1.

2.2. Selection of test conditions

The principles for the selection of test conditions for the preparation of extracts suitable for the *in vitro* toxicological assessment of paper and board samples are described in Bradley et al. (in preparation). In short, (a) the identities of the substances in extracts prepared for toxicological assessment should be related to the chemical migration from that sample into food, (b) the concentration of the substances in the extracts should be greater than or equal to migration into foodstuffs and, finally, (c) the extracts themselves should be compatible with the bioassay procedures. To achieve these three guiding principles, extraction and migration test conditions were proposed based on the product end use. The test conditions selected are shown along with the sample descriptions provided in Table 1. The non-food grade sample, NSP4, was initially described as being intended for contact with fatty foods and so it was extracted using ethanol. It was later revealed that this sample was a non-food grade product and it was then also extracted with water and exposed to Tenax for comparison purposes.

2.3. Extraction

The methods of extraction and migration testing are described in Bradley et al. (in preparation) and are in preparation as a CEN standard (CEN, 2007).

2.3.1. Water extraction

Water extracts were prepared for the six samples that were described as being intended for moist or wet food contact applications (NSP6, 7, 11, 18, 19 and 20) and subsequently for the non-food grade sample (NSP4). These water extracts were prepared by employing the CEN hot water or cold water extraction procedures, EN 647 and EN 645, respectively (CEN 1994a, 1994b) without any modification except for sterilisation of the water extract obtained. All water extracts were filter sterilised (0.22 μm) in order to avoid any microbial growth during transportation to the laboratories performing the bioassays and further handling of the extract prior to toxicity testing. Sterile filtered water was also supplied as a procedural blank. A description of the hot and cold water extracts is given in Table 2.

2.3.2. Ethanol extraction

Ethanol extracts were prepared as described in Bradley et al. (in preparation). Procedural blank samples were also prepared for each batch. Being naturally sterile, the ethanol extracts did not need sterile-filtering or refrigeration between preparation and testing. A description of the ethanol extracts is given in Table 3. Before testing the extracts were concentrated 50-fold to compensate the low ethanol tolerance of the biological test systems used (see 2.6 and 2.7.2).

A low molecular weight fraction (LMWF) of the ethanolic extract of the non-food grade sample NSP4 was also prepared using an Ultra-filtration (UF) membrane of nominal 1000 Da cut-off (Pall_OMEGA TM membrane, diameter, 90 mm; pressure of UF, 1.8 bar; UF system, Amicon TCF2). A procedural blank sample (ethanol only, i.e. no contact with the NSP4 sample) was prepared in the same way.

2.3.3. Tenax extraction

Tenax is a simulant for dry foods. Because Tenax is a finely-powdered insoluble polymer, it is necessary to take the Tenax after exposure to paper/board and extract the total migrate from it using a suitable solvent that can then serve to transfer the total migrate into the *in vitro* toxicity test systems. Ethanol was selected. The exposure, extraction and concentration methods are described elsewhere (Bradley et al., in preparation). For each batch of Tenax tests, unexposed Tenax was similarly extracted with ethanol and this was supplied as a procedural blank. A description of the concentrated ethanol extracts of the Tenax is given in Table 4.

2.4. Determination of the dry matter content

The dry matter content (DMC) of the water and ethanol extracts and of the low molecular weight fraction of the ethanol extract of NSP4 was determined. Known volumes of the extracts and the corresponding blanks were evaporated to dryness and the DMC was calculated gravimetrically. The Tenax extracts in an ethanol vehicle were not analysed for DMC. Because migration into this dry powdered polymer occurs mainly through the gas phase and therefore mainly volatile substances

Table 2
Description of aqueous extracts and the quantity of extractable matter (dry matter content, DMC) obtained

Code	Grammage (g/m^2)	Simulant and test conditions	Paper equivalents (g/ml water)	Paper equivalents (dm^2/ml water)	Extractable matter (mg/kg P/B)	Extractable matter (mg/dm^2 P/B)
NSP4	300	Water – 24 h at 20 °C	0.040	0.014	3300	9.2
NSP6	300	Water – 24 h at 20 °C	0.040	0.013	6100	17.1
NSP7	285	Water – 24 h at 20 °C	0.040	0.014	1200	3.2
NSP11	210	Water – 2 h at 80 °C	0.040	0.019	11,800	23.1
NSP18	240	Water – 24 h at 20 °C	0.040	0.017	7800	18.7
NSP19	300	Water – 24 h at 20 °C	0.040	0.013	3300	9.6
NSP20	30	Water – 24 h at 20 °C	0.040	0.130	2800	0.8

Table 3
Description of the 95% ethanol extracts and the quantity of extractable matter (dry matter content, DMC) obtained

Code	Grammage (g/m^2)	Simulant and test conditions	Paper equivalents (g/ml ethanol)	Paper equivalents (dm^2/ml ethanol)	Extractable matter (mg/l solvent)	Extractable matter (mg/kg P/B)	Extractable matter (mg/dm^2 P/B)
NSP4	300	95% ethanol – 24 h at room temperature	1.00	0.333	5190	5190	15.6
NSP5	500	95% ethanol – 24 h at room temperature	1.00	0.200	7070	7070	35.4
NSP8	140	95% ethanol – 24 h at room temperature	1.00	0.714	1340	1340	1.9
NSP13	666	95% ethanol – 24 h at room temperature	1.00	0.150	3800	3800	25.3

Table 4
Description of the ethanol extracts of the exposed Tenax

Code	Grammage (g/m^2)	Simulant and test conditions	Paper equivalents (g/ml ethanol)	Paper equivalents (dm^2/ml ethanol)
NSP1	665	24 h at room temperature	1.50	0.225
NSP2	300	5 days at 50 °C	0.675	0.225
NSP3	300	5 days at 50 °C	0.675	0.225
NSP9	170	5 days at 50 °C	0.383	0.225
NSP10	140	10 days at 20 °C	0.315	0.225
NSP12	105	5 days at 50 °C	0.236	0.225
NSP14	500	10 days at 20 °C	1.13	0.225
NSP15	70	5 days at 50 °C	0.158	0.225
NSP16	40	24 h at room temperature	0.090	0.225
NSP17	255	5 days at 50 °C	0.574	0.225

transfer, the gravimetric weight after removal of solvent by evaporation was not considered to be a reliable indicator of the mass of total migrate.

2.5. GC–MS analysis

2.5.1. Water extracts

For GC–MS analysis a portion (10 ml) of the water extract was placed in a 20 ml glass vial two internal standards added (14-methylpentadecanoic acid and cholestanol, 20 μg each). The water was then removed by evaporation to near dryness by employing a gentle stream of nitrogen gas. The residue was then re-dissolved in dry acetone (1 ml) and derivatised by adding the silylation reagent BSTFA (*N*,*O*-bis(trimethylsilyl)trifluoro-acetamide, 0.7 ml) and heating (70 °C, 30 min). The solvent and excess derivatisation reagent were removed by evaporation under a nitrogen gas stream, the residue dissolved in dichloromethane (1 ml) and then analysed by GC–MS using a procedure described by Bjorklund Jansson et al., 2002. The GC–MS instrument was equipped with a non-polar phase fused silica column (a VF-5 ms or a BPX5 column, 5% diphenyl – 95% dimethyl polysiloxane, 25 m × 0.25 mm i.d., 25 μm film thickness). The mass spectrometer (HP5989 MS-Engine) was operated with electron impact ionisation and run in full scan mode, m/z 50–600.

2.5.2. Ethanol extracts

The ethanol extracts were analysed by GC–MS both with and without derivatisation using BSTFA. Procedural blanks were analysed in both cases.

2.5.2.1. Direct GC–MS analysis. A portion of the extract (100 μl) was diluted to 1 ml with ethanol. Internal standards (1,9-dichlorononane and 1-fluorononane) were added at 20 μg/ml and the solution was analysed by GC–MS. The instrument used was an Agilent MSD 5973 inert, fitted with a DB-5 ms column (5% diphenyl – 95% dimethyl polysiloxane, 30 m × 0.25 mm i.d., 25 μm film thickness) and operated with EI ionisation and in full scan mode (m/z 40–450). The column was held at 40 °C for 3 min, raised to 280 °C at 10 °C/min, where it was held for 5 min. The injection (1 μl) was splitless (1 min) at 250 °C, with a transfer line temperature of 280 °C.

2.5.2.2. Derivatisation followed by GC–MS analysis. A portion (1 ml) of extract was placed into a 20 ml glass vial. Internal standard was added (hexadecanoic acid, 20 μl of a 2 mg/ml solution). The vial was placed under nitrogen flow to evaporate to near dryness. The residue was dissolved in acetone and then derivatised and analysed using GC–MS, as described above (Section 2.5.2.1).

2.5.3. Ethanol extracts of the exposed Tenax

The Tenax extracts in an ethanol vehicle were analysed by GC–MS following the addition of the internal standards (1,9-dichlorononane and 1-fluorononane, 2 μg/ml). The GC–MS conditions were as described above (Section 2.5.2.1). The extracts were also derivatised using BSTFA and the resulting solutions analysed by GC–MS as described above.

2.6. Cytotoxicity tests

2.6.1. 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) using the total protein content (TPC) as the endpoint. With HEp-2 cells also the neutral red uptake (NRU) was measured. Briefly, the cells grown in appropriate media and culture conditions, were seeded in 96 well microplates, and when either confluent (HEp-2 cells) or semiconfluent (Hepa-1c1c7 cells) they were treated with the test samples. With the water extracts this was done simply by reconstituting the culture medium in the extract resulting in the maximal sample concentrations of 80% (HEp-2 cells) or 90% (Hepa-1c1c7 cells). With the ethanol extracts the maximum concentration tolerated by the test system was 2% (v/v). Twofold serial dilutions of these top concentrations were done to determine the EC_{20} and EC_{50} values of the sample. The TPC determination was done after exposures of 24 and 72 h using either folin ciocalteau reagent (HEp-2 cells) or fluorescamine reagent (Hepa-1c1c7 cells) and a bovine serum albumin standard curve. The NRU determination was done as described by Borenfreund and Puerner (1985). A decrease of more than 20% in either TPC or NRU in comparison to controls was considered toxic.

2.6.2. The RNA synthesis inhibition test with human HepG2 and HeLa cell lines

The *in vitro* RNA synthesis inhibition was measured as a 30 min kinetic uptake of tritiated uridine into the cellular macromolecules as described by Fauris et al. (1985) and Valentin et al. (2001). The human cell lines used were a metabolically competent hepatic cell line HepG2 and a cervical cancer cell line HeLa. The automated procedures described by Severin et al. (2005) were used for both the cell types. Again, the water extracts were used to reconstitute the growth medium resulting in the maximal concentrations of 80%, while up to 2% (v/v) ethanol extracts were used with the HepG2 cells and 0.5% (v/v) with HeLa cells. A >40% but <70% decrease of RNA synthesis in comparison to controls was considered marginally positive, while a >70% decrease was considered clearly toxic.

2.6.3. The Inhibition of boar spermatozoan motility

Post exposure inhibition of sperm motility was assessed as described by Hoornstra et al. (2003). The extended boar semen, a commercial product obtained from an artificial insemination station (containing 27×10^6 spermatozoa/ml) was exposed to 1 vol.% of the test extract or its dilutions for 24–72 h at room temperature. The dilutions completely inhibiting the movement of the sperm cells were considered toxic.

2.7. Genotoxicity tests

2.7.1. Ames test

The standard Ames test (Maron and Ames, 1983) based on histidine auxotrophic *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA97 was applied to the samples. The S-9 preparation for metabolic activation system was either a commercial preparation (IFFA CREDO, L'Arbresle, France) or 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).

2.7.2. Comet assay with HepG2 cells

The *in vitro* SCG/Comet assay is a short-term test to study the induction of DNA damage in cultured cells (Uhl et al., 2000). Since HepG2 cells are metabolically competent, no metabolic activation was needed. 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 (Eagle's minimum essential medium supplemented with 10% fetal bovine serum (FBS), 1% MEM non-essential amino acid solution, 2 mM L-glutamine). After 20 h of incubation, the cells were treated with the test substance, the negative control (water or maximum 2% ethanol) and the positive control chemical (B[a]P 25 μM) for 20 h in 0.1 ml of the medium supplemented with 0.5% FBS. The water extracts were tested using the top concentrations, obtained by reconstituting the medium into the extract, while 2% (v/v) maximum concentrations of the extracts in ethanol (ethanol and Tenax extracts) were applied. At least three adequately spaced concentrations of the test substance were used selecting the highest concentration so that viability, as compared to the control cultures, was not decreased by more than 30%.

After the exposure, the cells were washed and harvested with trypsin–EDTA. Six wells treated with the same concentration were pooled, centrifuged, and resuspended in phosphate-buffered saline solution. Twenty microliter of cells (10,000 cells) were mixed in 75 μl of low-melting point agarose, spread on slides precoated with normal melting point agarose and lysed in ice-cold lysing solution. An electrophoresis was done, 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 the DNA damage in individual cells. The 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.

3. Results

3.1. Chemical composition of the extracts

None of the water or Tenax extracts gave a positive response in any of the bioassay procedures applied (see below). Nevertheless it is arguably just as important to know which paper extractables are non-toxic using the bioassay procedures applied, as it is to know what substances may elicit a positive, toxic, response. So the description here of the chemical composition of the water and Tenax extracts will be abbreviated, while more attention is paid to the ethanol extracts that were the most active in the bioassays.

3.1.1. Water extracts

The DMC of the seven water extracts is reported in Table 2. For the six cold water extracts the DMC was in the range 48–312 mg/l which is equivalent to between 0.8 and 18.7 mg/dm^2 when calculated on the basis of the paper/board surface area intended for moist or wet food contact. The DMC of the hot water extract of NSP18 was 472 mg/l which is equivalent to 23.1 mg/dm^2.

Many different substances were identified by GC–MS analysis of the derivatised water extracts from five of the paper/board samples NSP4, NSP6, NSP7, NSP19 and NSP20. In contrast, only a few substances were identified in the derivatised water extracts of samples NSP11 and NSP18. Fig. 1 depicts the quantities of identified substances summarised into different classes of compound as well as the sum total of the substances quantified.

The best library matches for the substances detected at concentrations in excess of 10 μg/dm^2 for each sample are described below. Other substances were detected below this concentration in all seven samples. Identification was achieved by comparison of the mass spectra obtained with library spectra present in an inhouse MS-library database at STFI Packforsk. In certain cases, e.g. for many of the fatty and resin acids, the library match identifications were confirmed by separate GC–MS analysis of the authentic standards. The concentrations were estimated by comparing the peak area of the identified substance with that of the nearest internal standard in the MS-chromatogram and assuming equal response factors.

NSP4: Two resin acids, dehydroabietic acid (151 μg/dm^2) and abietic acid (16 μg/dm^2), two fatty acids, nonanoic acid (19 μg/dm^2) and lauric acid (16 μg/dm^2) several hydroxyl and dicarboxylic acids, 3-hydroxypropanoic acid (34 μg/dm^2), 3,4-di-hydroxybutanoic acid (20 μg/dm^2), hydroxybutanedioic acid (17 μg/dm^2), an aromatic acid vanillic acid (14 μg/dm^2), glycerol (61 μg/dm^2), boric acid (32 μg/dm^2), and two lignans tentatively identified as conidendrinic acid isomers (12 μg/dm^2 and 30 μg/dm^2) were detected in the extracts in excess of 10 μg/dm^2. Other substances ($n = 13$) were detected below this concentration.

NSP6: Dehydroabietic acid (152 μg/dm^2), and abietic acid (55 μg/dm^2), isopimaric acid (17 μg/dm^2), two other unspecified resin acid isomers (15 and 12 μg/dm^2) and a sugar alditol (10 μg/dm^2) were detected in the extracts in excess of 10 μg/dm^2. Other substances ($n = 18$) were detected below this concentration. For this sample the total quantity of resin acids accounted for the major part, approximately 80%, of the whole GC–MS identifiable fraction.

NSP7: Glycerol (37 μg/dm^2), boric acid (27 μg/dm^2), hydroxyacetic acid (24 μg/dm^2), 3,4-di-hydroxybutanoic acid (21 μg/dm^2), hydroxybutandioic acid (21 μg/dm^2), 2-methyl-4-keto-pentane-2-ol (15 μg/dm^2), oxalic acid (11 μg/dm^2) and dehydroabietic acid (10 μg/dm^2) were detected in the extracts at concentrations in excess of 10 μg/dm^2. Other substances ($n = 25$) were detected below this concentration.

NSP11: Only two substances, a methylsiloxane oligomer (15 μg/dm^2) and ethylene glycol (12 μg/dm^2) were detected above 10 μg/dm^2. Other substances ($n = 2$) were detected below this concentration.

NSP18: Ethyleneglycol (46 μg/dm^2), a methylsiloxane oligomer (11 μg/dm^2) and oleic acid (11 μg/dm^2) were detected in the extracts above 10 μg/dm^2. Other substances ($n = 2$) were detected below this concentration.

NSP19: Boric acid (197 μg/dm^2), abietic acid (168 μg/dm^2), dehydroabietic acid (159 μg/dm^2), four unspecified resin acid isomers (82, 58, 19 and 12 μg/dm^2), triethyleneglycol (54 μg/dm^2), glycerol (52 μg/dm^2), neoabietic acid (42 μg/dm^2), 2-methyl-4-keto-pentane-2-ol (37 μg/dm^2), oxalic acid (30 μg/dm^2), hydroxyacetic acid (29 μg/dm^2), 2-hydroxypropanoic acid (28 μg/dm^2), 2,3-dihydroxypropanoic acid (11 μg/dm^2) and tetraethylene glycol (12 μg/dm^2) were detected at concentrations in excess of 10 μg/dm^2. Other substances ($n = 11$) were detected below this concentration. In this case the total quantity of resin acids constituted more than 50% of the whole GC–MS identifiable extract fraction (Fig. 1).

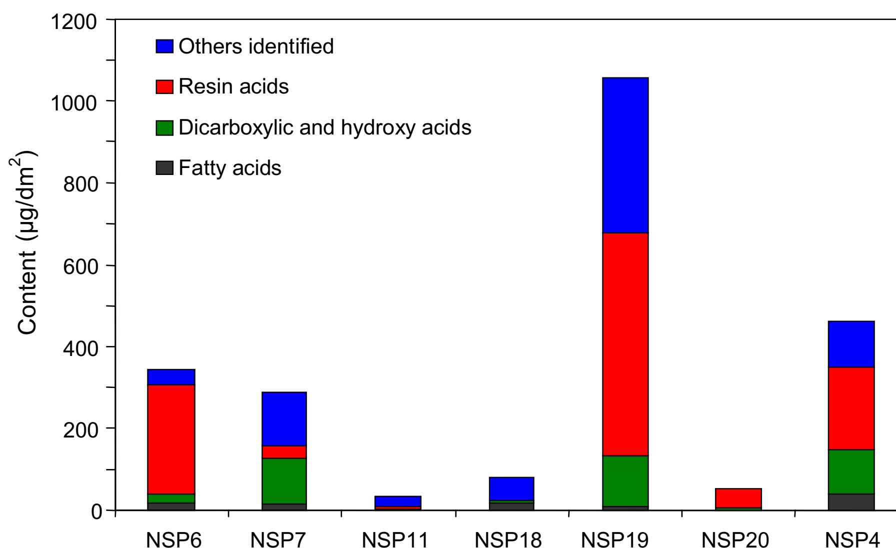

Fig. 1. Quantities of the different compound classes detected in the water extracts from paper and board samples (NSP 6, NSP 7, NSP 11, NSP 18, NSP 19, NSP 20 and NSP 4).

NSP20: Dehydroabietic acid (20 μg/dm^2) and abietic acid (12 μg/dm^2) were detected at concentrations in excess of 10 μg/dm^2. Other substances ($n = 20$) were detected below this concentration.

3.1.2. Ethanol extracts of the exposed Tenax

As discussed earlier DMC was not determined for the ethanol extracts of the Tenax, but they were subjected directly to GC–MS analysis. Although a number of substances were found to migrate the estimated levels were much lower than for the ethanol extracted samples (see later). Substances detected in the extracts at concentrations in excess of 10 μg/dm^2 are listed in Table 5.

3.1.3. Ethanol extracts

The DMC of the ethanol extracts are shown in Table 3. The DMC of the extracts supplied for application in the bioassays were in the range 1340–7070 mg/l.

A number of substances were detected in the ethanol extracts of paper/board samples. A number of trimethylsilyl esters were detected in the derivatised ethanolic extracts of the four samples. However, their identities were consistent with esters of those acids detected in the direct analysis of the concentrated ethanol extracts and therefore no additional information was derived. The substances found in the GC–MS analysis were tentatively identified by comparison with library spectra and their estimated concentrations are shown in Tables 6a–c. This table is organised as follows.

Table 6a lists all the substances found by GC–MS in the ethanol extract of NSP4, in rank order, and lists the occurrence or not of the same substances in the three other ethanol extracts, of NSP5, 8 and 13, for side-by-side comparison.

The next part, Table 6b lists the remaining substances detected in the extract from NSP5. Again, the table then lists the occurrence or not of the same substances in the other ethanol extracts, for side-by-side comparison.

Finally, the third part, Table 6c lists all the remaining substances. Clearly, from the forgoing description, those remaining substances were found in NSP8 or 13 only. These two extracts were not genotoxic and were only marginally cytotoxic.

The extract of NSP4 was the only sample showing positive results in genotoxicity assays and was also clearly cytotoxic (see Section 3.3.3). Its ethanol extract showed a total of 40 different GC–MS peaks which allowing for isomers (e.g. DIPN) and homologues (e.g. alkanes) gave the 27 entries in Table 6a amounting to a total of 757 μg/dm^2. DIPN, alkanes and phthalates were prominent. In retrospect, NSP4 turned out to be a non-food grade material that was introduced into the test programme as a 'blind' worst case sample. It had a 100% recycled content (Table 1).

Table 6a
Estimated concentrations (units of μg/ml of extract, also mg/kg paper since 1 g → 1 ml) of the substances in the ethanol extracts

RT (min)	NSP4	NSP5	NSP8	NSP13	Substance ID
	Yes	Yes	Marginal	Marginal	Extract is cytotoxic?
	Yes	No	No	No	Extract is genotoxic?
18.16/18.7	218	37		2	Diisopropylnaphthalene isomers
16–29.4	154	116		17	c15-29 *n*-alkanes
20.13	120	65	2.2	13	Diisobutyl phthalate
25.48	38	21		2.8	Dehydroabietic acid
21.1	37	10			Dibutyl phthalate
26.15	29	20	0.7	5.8	Bis(2-ethylhexyl) phthalate
19.02	27				Tetramethyl biphenyl isomer
23.6	22				Bis(2-ethylhexyl) fumarate
22.81	21	28		17	9-Octadecenoic acid
24.66	16	13	1.1	2.9	Methyl dehydroabietate
22.48	16	4.1			Octadecenoic acid, methyl ester
23.18	16			0.5	Bisphenol A
23.02	6.7	3.9			Octadecanoic acid
16.25	5.6	9.3			2-Phenylphenol
25.74	5.1				2-(Methoxymethyl)-2-phenyl-1,3-dioxolane
23.14	4.6				2-(Phenylmethoxy)naphthalene
17.11	3.1	3.4			Diethyl phthalate
26.58	2.9	3.5			7-Oxodehydroabietic acid, methyl ester
23.79	2.6				4-Benzyl biphenyl
14.72	2.4	3.9		12	Vanillin
17.68	2.4	2.6			Benzophenone
26.05	2.4				Dicyclohexyl phthalate
16.09	1.9	1.1			No library match
10.49	1.7				Nonanal
6.06	0.6	0.9	0.2	0.5	Hexanal
10.54	0.6				No library match
9.29	0.5	1.2			2-Ethyl-1-hexanol
	757	344	4.2	74	SUM

Table listing for all substances found in extract NSP4, in descending order of concentration.

NSP5 was not genotoxic but was clearly cytotoxic (see Section 3.3.3). The substances identified in this extract are listed in Tables 6a and b. Several perfluoro compounds were detected. When the identity of the paper/board samples was declared after testing, it became clear that sample NSP5 contained perfluorinated chemicals used to provide grease-resistance. This treatment was said by the

Table 5
Estimated concentrations (μg/dm^2) of the substances in the Tenax extracts

	NSP1	NSP2	NSP3	NSP4	NSP9	NSP10	NSP12	NSP14	NSP15	NSP16	NSP17
Extract is cytotoxic?	No	No	No	No	No	No	No	No	No	No	No
Extract is genotoxic?	No	No	No	No	No	No	No	No	No	No	No
C16-27 *n*-alkanes		143	101	186	88	22	107	38			
DIPN isomers	32	140	46	200	17	120	22	110			
Diisobutyl phthalate		110	28		73	61	50				
Methyl octadecenoate		32	18							41	
Dibutyl phthalate		31		77		10	21	98			
Bis(2-ethylhexyl) hexadecanoate		17									
Bis(2-ethylhexyl) phthalate		13	23				66		40		
Methyl dehydroabietate		12									18
Dicyclohexyl phthalate		10									
Unknowns			12								18, 11
Tetramethyl biphenyl isomer				18		16					
Hexanal											12
Octadecene											39
1H-naphtho(2,1-b)pyran, 3-ethenyl-dodecahydro-3,4a,7,7,10a-pentamethyl-,(3S-(3. α.,4a. α,6a. β, 10a. α, 10b. β)											12
SUM	32	508	228	481	178	229	266	246	40	41	110

Table 6b
Estimated concentrations (units of μg/ml of extract, also mg/kg paper since 1 g → 1 ml) of the substances in the ethanol extracts

RT (min)	NSP4	NSP5	NSP8	NSP13	Substance ID
	Yes	Yes	Marginal	Marginal	Extract is cytotoxic?
	Yes	No	No	No	Extract is genotoxic?
9.44		290			1-Methyl-2-pyrolidinone
25.87		82	11	49	No library match
24.17		49	7.8	13	Eicosene
22.34		48			No library match
21.71		31			1-Cyclohexene-1-carboxylic acid, 4-(1,5-dimethyl-3-oxohexyl)-, methyl ester [S-(R*,R*)]
23.6		11		2.4	Bis(2-ethylhexyl)fumarate
28.46		11	29	15	Stigmast-7-en-3-ol, (3. β.,5. α.,24S)
22.76		5.2		4.3	9,12-Octadecedienoic acid
24.37		5.1		1.3	No library match
22.17		4.6			1-Naphthalenepropanol, α.-ethenyldecahydro-. α.,5,5,8a-tetramethyl-2-methylene-, [1S-(1. α.(S*),4a.β.,8a.α.)]
26.2		3			15-Hydroxydehydroabietic acid, methyl ester
22.7		2.6			No library match
16.72		2.1			Dodecanoic acid
7.76		2			Perfluoro compound
9.22		1.5			Perfluoro compound
22.98		1.4			No library match
13.58		1.2			Perfluoro compound
9.95		1.1			Acetophenone
12.05		1			Perfluoro compound
6.2		0.6			Perfluoro compound
12.4		0.6			No library match
12.6		0.4			No library match
12.17		0.3			No library match
12.28		0.3			No library match
8.25		0.2			No library match
	0	555	48	85	SUM

Table listing for all other substances found in extract NSP5, in descending order of concentration.

Table 6c
Estimated concentrations (units of μg/ml of extract, also mg/kg paper since 1 g → 1 ml) of the substances in the ethanol extracts

RT (min)	NSP4	NSP5	NSP8	NSP13	Substance ID
	Yes	Yes	Marginal	Marginal	Extract is cytotoxic?
	Yes	No	No	No	Extract is genotoxic?
22.34			5.1	28	Octadecene
21.11				8.5	No library match
17.2				5.6	2,6-Dimethoxy-4-(2-propenyl)phenol
21.82				3.8	1H-naphtho(2,1-b)pyran, 3-ethenyldodecahydro-3,4a,7,7,10a-pentamethyl-, [3R-(3. α.,4a. β.,6a. α., 10a. β., 10b. α.)]
27.11				3.8	No library match
17.89				3.6	4-Hydroxy-3,5-dimethoxybenzaldehyde
24.04			1.1	3.5	No library match
23.75			2.8	2.6	4-(2-(4-Nitrophenyl)ethylbenzamine)
28.76			7.7	2.4	No library match
23.26				1.7	Pregn-14-ene, (5. β.)
15.79				1.1	1-(3-Hydroxy-4-methoxyphenyl)ethanone
18.39				0.8	No library match
7.19				0.4	No library match
26.39			6.4		γ-ergostanol
30.79			3.8		No library match
26.24			1.3		2,2′-Bis(*p*-methoxyphenyl)-1,1-Dichloroethylene
23.26			0.8		No library match
	0	0	29	66	SUM
	757	899	81	225	SUM ALL, Tables 6a–c

Table listing for all remaining substances, in descending order of concentration in NSP13.

manufacturer to be incompatible with ethanol and so an alternative fat simulant would have to be used for testing. In testing plastics the usual alternative to ethanol is isooctane although this solvent has very different polarity. In testing polymeric coatings on metal substrates ('can coatings') the extractant used commonly is acetonitrile. As a non-hydroxylic solvent, acetonitrile may be a suitable alternative stimulant for paper although it may be rather toxic to some of the cell lines used subsequently. This should be investigated further.

3.2. Comparing the GC–MS data with the dry matter content

The sum of all substances estimated in the GC–MS analysis is compared with the gravimetric dry matter content obtained by evaporation of the water and concentrated ethanol extracts in Table 7. In all cases only a small proportion (0.2–11% for the water extracts and 6.8–15% for the ethanol extracts) of the extractable matter could be detected and estimated using GC–MS. Much of this missing fraction is expected to be made up of substances of high molecular weight which are not sufficiently volatile to be detected using GC–MS. Analysis by LC–MS was not carried out as there are no searchable mass spectral libraries to facilitate peak identification. However, it cannot be ruled out that other substances exist in the extracts that have molecular weights of less than 1000 Da, and thereby have the potential to be toxicologically significant, but are not amenable to analysis by GC–MS even after derivatisation. In addition the identities of some of the ethanol extractable substances did not provide good library matches. Therefore any toxicity associated with these unknowns cannot be considered by simply analysing the extracts in this way. This helps to illustrate the need for a global bioassay assessment of the safety of the total migrate from paper and board, which chemical analysis alone cannot provide.

3.2.1. Low molecular weight fraction

Once passed through the 1000 Da membrane the DMC of the ethanol extract of NSP4 decreased to 133 mg/l which is 6.65 mg of dry matter for the 50 ml fraction that was collected. This is equivalent to 138 mg/kg of board. The unfractionated DMC for NSP4 was 5190 mg/kg (Table 3) so the LMWF after drying contained just 2.7% of the total extractable DMC.

The opposite was true for the low molecular weight fraction for which the sum of GC–MS results is 764 mg/l whereas the DMC measured after evaporation of this sample was only 130 ± 30 mg/l. This reveals two things: (a) the UF procedure not only has retained the LMWF but it has successfully isolated it from the HMWF (b) evaporation of the LMWF leads to significant loss by volatilisation.

The substances detected in the LMWF of NSP4 using GC–MS after subtraction of the procedural blank are shown in Table 8.

Table 7
Comparing the sum of the GC–MS extractable substances with the DMC

Code	Extraction medium	Sum of the GC–MS extractables (mg/dm^2)	Dry matter content (mg/dm^2)	DMC accounted for by GC–MS (%)
NSP4	Water	1.20	9.2	14.1
NSP4	95% ethanol	2.27	15.6	14.6
NSP5	95% ethanol	5.19	35.5	14.6
NSP6	Water	0.35	17.1	2.0
NSP7	Water	0.29	3.2	9.1
NSP8	95% ethanol	0.13	1.8	7.2
NSP11	Water	0.04	23.1	0.2
NSP13	95% ethanol	1.71	25.3	6.8
NSP18	Water	0.08	18.7	0.4
NSP19	Water	1.06	9.6	11.0
NSP20	Water	0.05	0.8	6.8

Table 8
Estimated concentrations of the substances in the low molecular weight fraction of the ethanol extract of NSP4

RT (min)	μg/ml	Best library match
18.6–19.2	201	Diisopropylnaphthalene isomers
20.55	100	Diisobutyl Phthalate[a]
23.50	100	Ethyl octadecenoate[a]
13.77	78	Siloxane
21.87	71	Ethyl hexadecanoate[a]
15.97	49	Siloxane
26.56	41	Di-(2-ethylhexyl) phthalate[a]
23.58	38	Bisphenol A[a]
21.52	21	Dibutyl phthalate[a]
19.46	27	Tetramethyl biphenyl isomer
17.94	17	Siloxane
19.83	13	Ethyl tetradecanoate
25.09	8	Dehydroabietic acid, methyl ester
	764	SUM ALL

[a] Low levels detected in the blank too.

When compared with the unfractionated extract (Table 6a) it is clear that the LMWF has picked-up some extraneous siloxanes and bisphenol A, most probably from the materials used in the membrane fractionation apparatus or the membrane itself and that the levels in the sample were, by coincidence, higher than in the procedural blank extracts. Also, some of the minor hydrocarbons (pentadecane, hexadecane, nonadecane, tricosane, tetracosane, etc.) were lost, most probably due to absorption of these non-polar substances out of the 95% ethanol and into the membrane or the plastics used in the apparatus. The LMWF was enriched in certain ethyl esters compared to the raw extract which had more free acids and methyl esters, most probably caused by esterification in the ethanol solvent during fractionation. These differences apart, the total concentration of GC–MS detectable substances are comparable (Tables 6a and 8) as are certain key substances of interest for paper that would be expected to pass through the membrane unchanged, such as the DIPN isomers which are present in the whole fraction at a concentration of 218 μg/ml and in the low molecular weight fraction at a concentration of 201 μg/ml. Similarly the concentrations of di(2-ethylhexyl) phthalate (29 versus 41 μg/ml), dibutyl phthalate (37 versus 21 μg/ml), and diisobutyl phthalate (120 versus 100 μg/ml), in the whole and low molecular weight fractions were similar.

3.3. Cytotoxicity and genotoxicity of the extracts

3.3.1. Water extracts

The water extracts of NSP7, NSP11, NSP19 and NSP20 were consistently non-toxic in the cytotoxicity assays and none of the extracts was positive in the genotoxicity tests (data not shown). Only with the NSP6 and NSP18 extracts there was an indication of reduced cellular viability of HEp-2 cells, and only in the NRU-assay (Fig. 2). This effect was not seen in the TPC-assay with HEp-2 cells (Fig. 3), nor in other cell lines or assays (i.e. Hepa-1c1c7 cells; Fig. 4).

3.3.2. Ethanol extracts of the Tenax

None of the extracts, even at the highest tolerated concentrations (2 vol.% of ethanol extract) applied, produced any positive

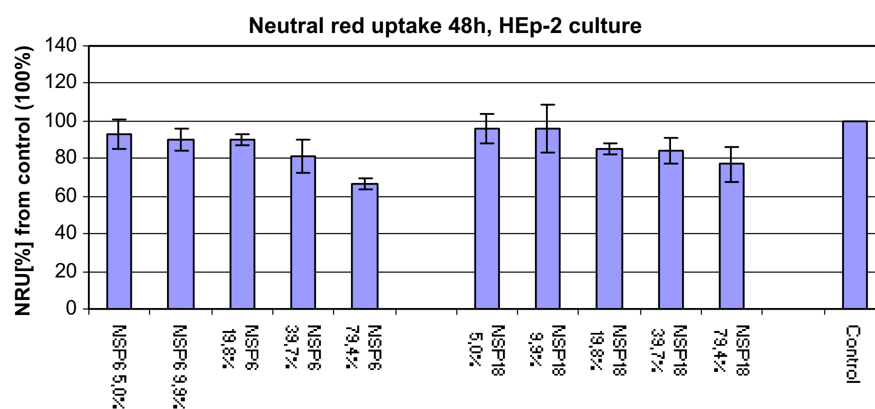

Fig. 2. The NRU-assay results of the NSP6 and NSP18 water extracts in HEp-2 cell culture. The bars indicate the standard deviation, SD.

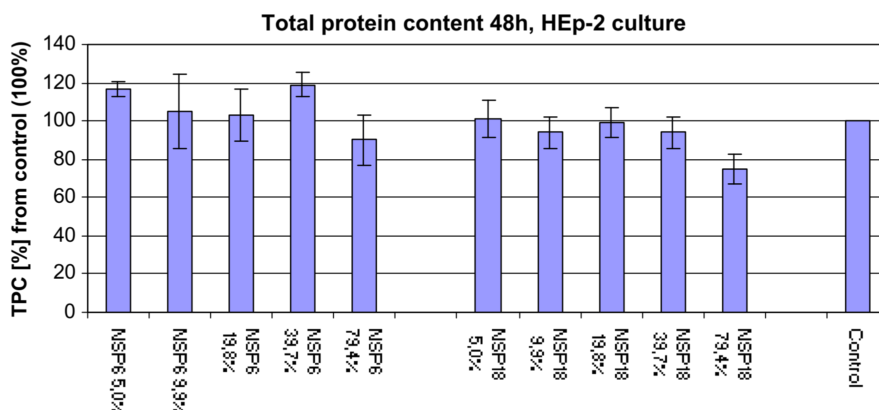

Fig. 3. The TPC results of the NSP6 and NSP18 water extracts in HEp-2 culture. The control represents the value of unexposed cell culture. The bars indicate the standard deviation (SD).

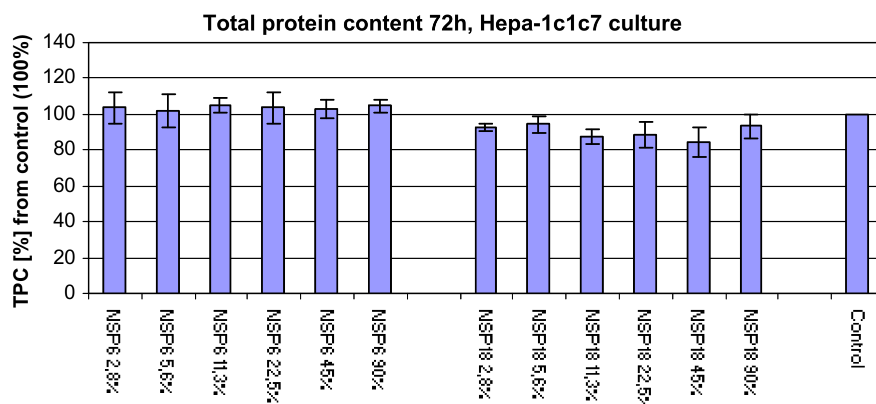

Fig. 4. The TPC results of NSP6 and NSP18 water extracts in Hepa-1c1c7 culture. The control represents the value of unexposed cell culture. The bars indicate the standard deviation (SD).

response either in the cytotoxicity or genotoxicity assays (data not shown).

3.3.3. Ethanol extracts

The results obtained with the ethanol extracts of the samples NSP4, NSP5, NSP8 and NSP13 are summarised in Tables 9 and 10. It can be seen, that NSP4 and NSP5 displayed conspicuous cytotoxicity, while only marginal effects could be seen with NSP8 and NSP13. The detailed results of acute cytotoxicity assays and RNA synthesis inhibition tests are presented in Figs. 5–7, and those of the boar spermatozoan motility inhibition assay in Table 10. The low molecular weight fraction of NSP4 (see Section 3.2.1) was tested for RNA synthesis-inhibition in HepG2 cells, but was not found cytotoxic (data not shown). NSP4 extract induced mutations in the Ames tester strain TA98 without metabolic activation (Table 11), but was negative in the Comet assay (Fig. 8). None of the other ethanol extracts was positive in either of the genotoxicity assays (data not shown).

Table 9
Cytotoxicity results of the paper and board samples representing contact with fatty foods and extracted with 95% ethanol using procedure with a high paper/board versus solvent ratio followed by a 10-fold concentration step

Code	Acute cytotoxicity[a], the highest tested concentration 2% (v/v)		Sublethal cytotoxicity RNA synthesis inhibition [b], the highest tested concentrations either 2% (v/v) for HepG2 cells or 0.5% (v/v) for HeLa cells	
	HEp-2 cells	Hepa-1c1c7 cells	HepG2 cells	HeLa cells
NSP4	EC_{50} 0.5 ± 0.1%	EC_{50} 0.44 ± 0.03%	EC_{50} 0.38 ± 0.03%	Synthesis down to 16 ± 7% of the control
NSP5	EC_{50} 0.6 ± 0.5%	EC_{50} 0.45 ± 0.01%	EC_{50} 0.29 ± 0.02%	Synthesis down to 9 ± 1% of the control
NSP8	EC_{20} 1.0 ± 0.2%	EC_{20} 2.0 ± 0.01%	EC_{50} 1.49 ± 0.12%	Synthesis down to 55 ± 3% of the control
NSP13	EC_{20} 0.8 ± 0.4%	EC_{50} 1.90 ± 0.1%	EC_{50} 0.92 ± 0.01%	Synthesis down to 34 ± 3% of the control

[a] Either the EC_{50} or EC_{20} values (with ±SDs) are given, depending which one was reached.
[b] EC_{50} values are given for HepG2 cells, with HeLa cells only a single concentration was tested, and the results indicate the level of the RNA synthesis observed (±SD).

Table 10
The sublethal toxicity of the samples NSP4, NSP5, NSP8 and NSP13 as indicated by results of the boar spermatozoan motility inhibition

	IC100 (vol.%) UKU		IC100 (vol.%) UH	
Code	1 day	3 day	1 day	4 day
NSP 4	0.25	0.25	0.2	0.1
NSP 5	0.25	0.25	0.2	0.1
NSP 8	1.00	0.50	0.5	0.5
NSP 13	0.50	0.50	0.5	0.2

University of Kuopio (UKU) tested the samples as such, University of Helsinki (UH) concentrated the samples 10 times (results are calculated to correspond the unconcentrated sample).

4. Discussion

Chemical analysis of extracts of 20 paper/board samples, prepared to mimic the worst case migration from the sample into a foodstuff, identified a range of substances. However, this analytical method was not capable of detecting and identifying all substances extracted/migrating from the paper/board samples. Using the concentrated ethanol extracts as an example if the sum of all substances estimated in the GC–MS analysis is compared with the gravimetric dry matter content obtained by evaporation it is apparent that only a small proportion, 6–15%, of the extractable matter can be detected and estimated using GC–MS. In addition some of the GC–MS peaks that were detected could not be identified clearly. Since the chemical identity of the majority of the compounds present in the migrates cannot be established, the safety assessment based on toxicological data on individual compounds and determination of the respective ADI or TDI values, is not feasible. This situation illustrates the need for a global assessment of the safety of the total migrates from paper and board.

The only markedly toxic samples were the concentrated ethanol extracts NSP4 and NSP5. NSP4, from which the most cytotoxic and genotoxic extract was prepared, represents a non-food grade material that was included as a worst case sample (although tested blindly). NSP5 turned to be out to be treated with fluorinated chemicals not compatible with the ethanol used in extraction. These two had also highest amount of identified compounds (757 μg/ml and 899 μg/ml) compared to 81 μg/ml and 255 μg/ml observed in NSP8 and NSP13, respectively. NSP8 and NSP13 showed marginal cytotoxicity. NSP4 contained relatively high amounts of DIPNs and diisobutyl phthalate (45% of the identified compounds, Table 6a). However, their concentrations in final test mixture are still less than 10% of the cytotoxic concentrations observed when these compounds have been tested as such (data not shown). In NSP5 the most abundant identified compound was 1-methyl-2-pyrrolidinone. Apparently no cytotoxicity studies on this compound have been published. It is, however, known to cause aneuploidy in yeast (Mayer et al., 1988). Also in NSP5, several perfluorinated compounds were identified at relatively low concentrations (1–2 μg/ml). While these compounds are suspected

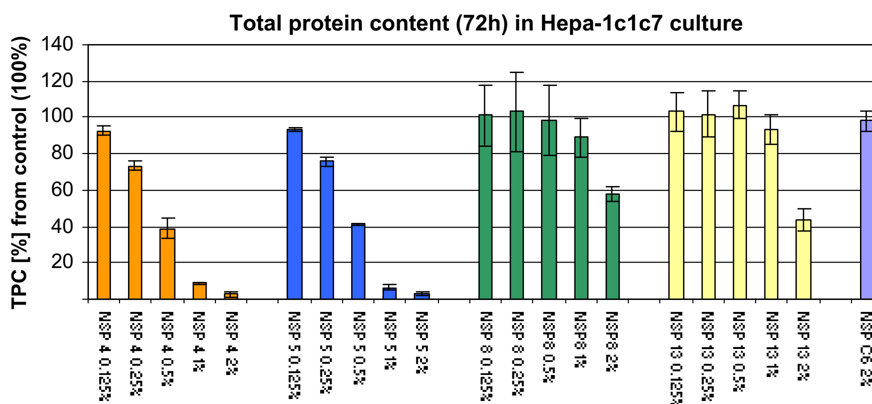

Fig. 5. Cytotoxicity of ethanol extracts of NSP4, 5, 8 and 13 in Hepa-1c1c7 culture. The control represents the value of cell culture exposed to 2% (v/v) of control ethanol. The bars indicate the standard deviation (SD).

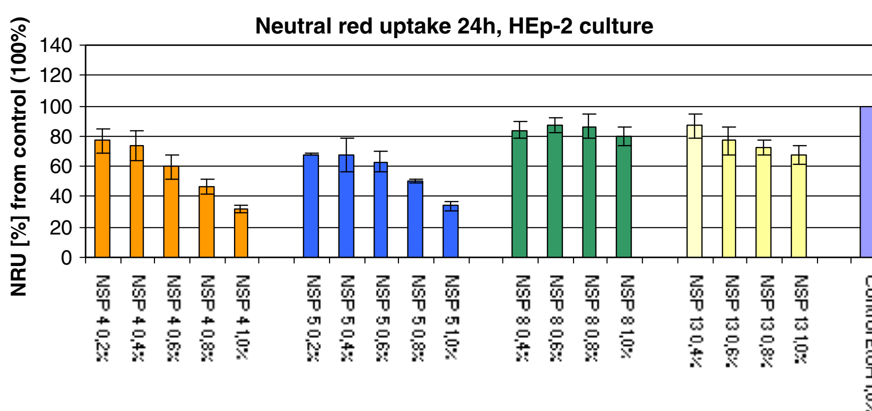

Fig. 6. NRU assay results of NSP4, 5, 8 and 13 ethanol extracts in HEp-2 culture. The control represents the value of cell culture exposed to 1% (v/v) of control ethanol. The bars indicate the standard deviation (SD).

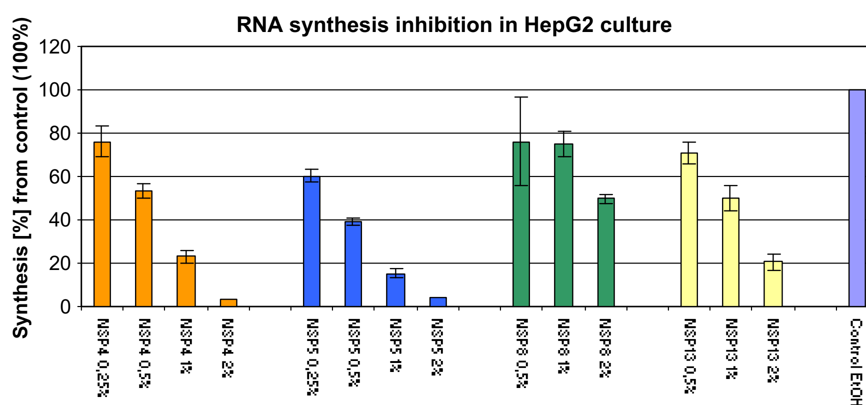

Fig. 7. Results of RNA synthesis inhibition assay of NSP4, 5, 8 and 13 ethanol extracts in HepG2 culture. The control represents the value of cell culture exposed to 2% (v/v) of control ethanol. The bars indicate the standard deviation (SD).

endocrine disrupters and have been indicated in developmental disorders (Maras et al., 2006; Lau et al., 2004) few studies have been published about their acute cytotoxicity. In a recent paper by Kleszczyński et al. (2007) the reported cytotoxic concentrations of perfluorinated carboxylic acids for different human cell lines were clearly higher (20 times or more) than the concentrations of perfluorinated compounds detected here.

Thus, among the identified compounds there appears to be no individual candidates that singly could account for the cytotoxicity effects observed with NSP4 and NSP5. Nor are there any obvious genotoxic compounds that could explain the positive Ames test result of NSP4. Since the presence of metabolic activation abolished the mutagenic activity, it is possible that the negative results of the comet assay on this sample reflect the detoxification by the liver-derived HepG2 cell line used in the test. It should be noted that identified compounds present only a fraction of total extractable matter in the extracts (Table 3).

While the results indicate a correlation between the total amount of extractables and cytotoxicity, also observed in earlier studies in paper and board toxicity (Fauris et al., 1998; Binderup

Table 11
The Ames test results of the sample NSP4 and control chemicals

Test agent	Concentration/plate	Number of revertants (mean of three plates ± SD)							
		TA1535		TA100		TA98		TA97	
		−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
Ethanol 94%	200 µl	24 ± 7	25 ± 4	141 ± 3	178 ± 10	26 ± 8	32 ± 6	102 ± 9	121 ± 7
Water	200 µl	14 ± 3	20 ± 2	nt	nt	nt	nt	nt	nt
Epichlorohydrin	1 µl	1807 ± 161	nt	nt	nt	nt	nt	nt	nt
2-Aminoantracene	1 µg	27 ± 3	736 ± 24	nt	nt	nt	ny	nt	nt
Benzo(a)pyrene	10 µg	nt	nt	152 ± 2	742 ± 15	20 ± 2	235 ± 21	122 ± 8	508 ± 25
Nitroquinolineoxide	1 µg	nt	nt	nt	nt	589 ± 23	nt	958 ± 56	135 ± 7
Sodiumazide	0.5 µg	nt	nt	850 ± 30	nt	nt	nt	nt	nt
NSP4	5 µl	nt	nt	nt	nt	26 ± 6	nt	nt	nt
NSP4	10 µl	nt	nt	nt	nt	33 ± 2	nt	nt	nt
NSP4	25 µl	nt	nt	nt	nt	41 ± 6	nt	nt	nt
NSP4	50 µl	25 ± 4	24 ± 5	33 ± 3	54 ± 12	70 ± 17	40 ± 9	119 ± 12	130 ± 6
NSP4	100 µl	18 ± 4	26 ± 2	35 ± 3	47 ± 7	90 ± 13	27 ± 8	126 ± 18	138 ± 15
NSP4	200 µl	23 ± 6	19 ± 4	27 ± 8	37 ± 6	114 ± 6	43 ± 6	108 ± 8	85 ± 12

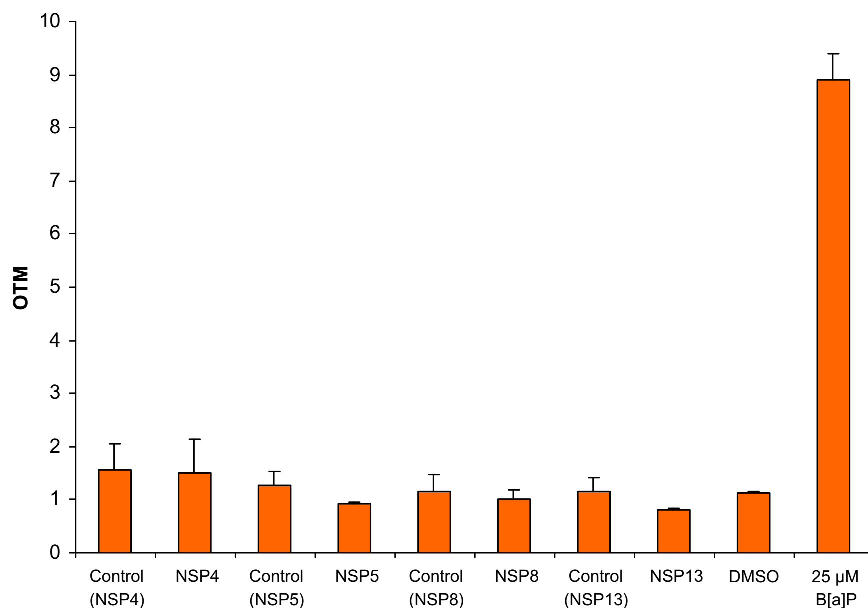

Fig. 8. Results of the Comet assay of NSP4, NSP5, NSP8, NSP13 ethanol extracts (two experiments). Ethanol controls were included for each assay. The bars indicate the standard deviation (SD).

et al., 2002), they also demonstrate the limitations in identifying potentially harmful compounds by chemical analysis alone. Not only does a major fraction remain unidentified, but synergistic toxic effects of different extractables probably have a role that is not apparent from consideration of the chemical composition alone. Also, as seen with the water extracts (Table 2 in comparison with Table 3) the amounts of extractable matter can be equal or even higher than in concentrated ethanol extracts. Thus the cytotoxicity does not simply depend on the quantity of the extractable substances but very specifically about their composition.

There was a remarkable correlation between the results obtained in the different cytotoxicity assays used in this study. The most clear difference between the responses of different cell lines was the considerably higher sensitivity of the HeLa cells than HepG2 cells in the RNA synthesis inhibition assay (Table 9). This might reflect the different metabolic potential of the cell lines, HepG2 being probably able to deactivate toxic substances present in the extract.

Altogether, the cytotoxicity test battery used combines several relevant toxicological endpoints forming a continuum from acute cytotoxicity assays to RNA synthesis inhibition tests, both being a prerequisite for meaningful mammalian cell genotoxicity assays. The tests are relatively easy to perform and have easily observable endpoints making them useful for routine analysis of paper/board products. While there is an apparent flexibility in choosing cell lines for testing of paper and board, the inclusion of metabolically competent hepatic cell lines is advisable in order to detect both the eventual detoxification and the possible activation of the toxic compounds present in the sample. The hepatic cell lines also provide some possibilities to expand the toxicological endpoints of the battery, taking into account their ability to react with hormones, including synthetic estradiols, estrogens and androgens (Browne-Martin and Longcope, 2001; Tang et al., 2007). The induction of haepatic xenobiotic metabolism, easily measurable by 7-ethoxyresosufin-*O*-deethylase activity, by dioxins and dioxin like compounds at nanomolar concentrations (Silkworth et al., 2005) opens also possibilities to screen also for these types of compounds. As pointed out in the introduction, also specific bioassays for endocrine disruptors and dioxin exist and have been applied for paper and board extracts (Binderup et al., 2002) and could be easily integrated into the scheme.

The two genotoxicity assays used, Ames test and the comet assay fulfill the criteria of high-throughput, rapid tests suitable for screening purposes. The results of comet assay are in good agreement with more laborious cytogenetic assays, such as micronucleus test and chromosome aberration test (Hartmann et al., 2001, 2003), with the exception of agents acting primarily on the mitotic spindle.

We believe that short-term assays, though not validated tests for oral safety assessment, are a useful screening tool in the safety evaluation of chemically complex food contact materials. However, we emphasise that the conclusions about the safety of a tested material have to be made taking into account the actual end use and application. The procedures used to prepare the test extracts are worst case scenarios designed to maximise the exposure of the cells to the compounds derived from the sample. In the actual application the type of the food contact (direct, indirect) and the relevant conditions (temperature, time) may reduce the migration of cytotoxic compounds to a practically insignificant amount. There are no safety limits, at present, indicated for a material that exhibits a response to the genotoxicity assays, i.e. such materials should not be used in contact with food. If a material gives a positive response to one or more cytotoxicity assay then it should be considered whether or not the toxic principle in the extract under the known conditions of use would be expected to migrate to such extent into food that the actual concentration in the food would also result in the same cytotoxic response. In these cases the proper procedure is to identify the toxic compound(s) and subject them to a more thorough safety assessment. This could be done by applying the most sensitive bioassays to different fractions of the test material, thus pinpointing the toxicity to certain chemical or fraction that can be then characterized.

Conflict of Interest Statement

The authors declare that there are no Conflicts of Interest.

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