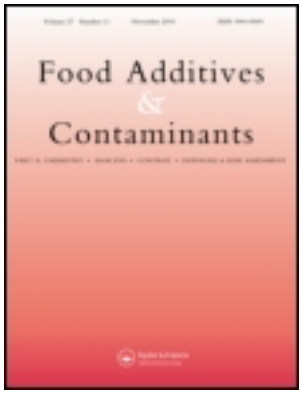

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Safety assessment of food-contact paper and board using a battery of short-term toxicity tests: European union BIOSAFEPAPER project

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Safety assessment of food-contact paper and board using a battery of short-term toxicity tests: European union BIOSAFEPAPER project

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Abstract
An European Union (EU)-funded project QLK1-CT-2001-00930 (BIOSAFEPAPER) involves the development, validation and intercalibration of a short-term battery of toxicological tests for the safety assessment of food-contact paper and board. Dissemination of the results to industry, legislators (e.g. DG Consumer Protection, DG Enterprises, DG Research), standardization bodies such as CEN, and consumers will create an agreed risk evaluation procedure. The project involves pre-normative research in order to establish a set of *in-vitro* cytotoxicity and genotoxicity tests that will be easily adaptable to food-contact fibre-based materials and have endpoints relevant to consumer safety, including sub-lethal cellular events. These tests will be performed on samples representing actual migration conditions from food-contact paper and board with respect to different foodstuffs, and should form an experimental basis for scientifically sound recommendations for a harmonized system of risk evaluation and product testing.

Keywords: *Cytotoxicity assays, extraction procedures, paper and board*

Introduction

One of the main objectives of the project, Application of Bioassays for Safety Assessment of Paper and Board for Food Contact (BIOSAFEPAPER, QLK1-CT-2001-00930), is the development of a battery of toxicological tests with a decision-tree approach with endpoints relevant to consumer safety. The project has a modular structure. Improved test systems measuring both acute and sub-lethal effects on mammalian cells, cross-validated by the project partners, are being evaluated for the eventual inclusion in the final test battery (Module I). Extraction procedures applicable to paper and board with solvent/adsorbent systems simulating different types of foodstuffs and suitable for the further biological *in-vitro* tests need to be assessed and also developed taking into account the end-use applications (Module II). Based on the scientific evaluation of the results, risk assessment procedures dedicated to paper and board in contact with food are being designed (Module III). Simultaneously, the results will be disseminated to consumers, legislators, standardization bodies such as CEN, and industry as an eventual basis for regulatory activities. So far, the expected achievements from this project are tools (analytical and biological) for ensuring the safety of food-contact paper and board products, harmonization of testing procedures applicable both to regulatory

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purposes, and for the hazard analyses approach in the industry.

According to the pre-normative nature of this research programme, the tests being developed should receive, at the end, regulatory acceptance based on scientific recognition and robustness demonstrated in different laboratories. The requirements of such an appropriate validation are indicated in several documents (Balls et al. 1990, 1995; Anon. 1995; OECD 1996; Balls and Fentem 1997). Usually, this validation involves an interlaboratory blind trial designed to demonstrate that the data generated can be adopted for decision-making purposes. The present paper should be considered as the sum of the results obtained at the mid-term of the project. Emphasis has been on the development of extraction procedures relevant for paper and board, and especially on the selection of cytotoxicity tests to be used in the second half of the project on actual paper and board samples.

In the extraction module, realistic extraction procedures will be developed for a further toxicological testing of paper and board. This will be done by using paper and board samples spiked with known toxic substances and applying different extraction strategies taking into account the requirements of different food matrices. When the extraction procedures have been tentatively introduced, they will be applied to actual paper and board samples. The extraction procedures must fulfil the following criteria:

- Identity: Chemical content should be related to the chemical migration determined for that paper sample in contact with foodstuffs.
- Concentration: Concentration in the extract should be no less than the concentration of migrants in foodstuffs.
- Compatibility: Extract should be homogenous, stable, free from particulates and suitable for subsequent bioassay procedures.

In this paper, the outcome of certain standardized extraction procedures applied to a number of paper and board model samples and the analysis of the extractants are described.

Regarding the toxicological tests, the criteria for acceptance are as follows:

- Sensitivity: Positive control substances will be detected at concentrations suitable for real-life control situations, i.e. for the most harmful substances, at migration or extraction levels that are equivalent to 'not detectable', $<10\,\mu g\,kg^{-1}$ in the diet.
- Consistency: Same tests give repeatedly qualitatively similar responses to positive controls and historical control samples in different participating laboratories.
- Discriminatory power: Responses reflect actual differences in the toxicities of the samples.

The *in-vitro* assays as screening tests for predicting toxicity of chemicals, or cellular and subcellular toxicity mechanisms, are receiving increasingly more attention, and their use is in the process of being consolidated in formal testing protocols (Broadhead and Combes 2001). Cells respond rapidly to a toxic stress by a decrease of metabolic activities, cell growth and gene transcription. Cytotoxicity has long been considered an indicator of the acute toxicity observed *in-vivo*. Many *in-vitro* cytotoxicity tests measure cell death and they are useful to define the intrinsic ability of a substance to cause the cell death (basal cytotoxicity) because of damage to the main cellular functions. Because a good correlation has been observed between *in-vitro* basal cytotoxicity and *in-vivo* acute toxicity in animals and humans (Clemedson et al. 2000), cytotoxicity tests are also used to define the concentration range for further targeted *in-vitro* tests as genotoxicity tests, and could reduce to a large extent the frequency of false-positive or -negative results. By selecting suitable endpoints, useful information of the cellular targets of toxic substances can also be obtained from a cytotoxicity test battery. Selected and often used endpoints include modification of the cellular permeability, impairment of the mitochondrial functions, alterations in the cellular morphology, and a decrease of cellular growth or inhibition of replication. Changes in membrane permeability can be measured by differential staining techniques (trypan blue exclusion, Phillips 1973; neutral red uptake, Borenfreund and Puerner 1984) or by the release of intracellular enzymes (lactate dehydrogenase; Korzeniewski and Callewaert 1983) or of preloaded ^{51}Cr (Holden et al. 1973). Uridine uptake (Shopsis and Sathe 1984; Valentin et al. 2001) and nucleoside release (Thelestam and Molby 1976) are also considered as markers for membrane integrity. Mitochondrial activity can be measured using the rhodamine 123 test (Rahn et al. 1991) or MTT-dye test (Mosmann 1983). Boar spermatozoan motility inhibition is also a highly specific indication of impairment of either mitochondrial activity or membrane integrity or both (Andersson et al. 1998). The cellular replication can be measured as colony-forming ability (Acosta et al. 1980; Wilson 1992), while the total protein content determination (Balls and Bridges 1984) and neutral red uptake (Borenfreund & Puerner 1985) are indicators of cellular viability. Whatever cytotoxicity endpoint is eventually used, special attention must be paid to

the *in-vitro* cellular system which is the most important technical aspect of the test. Most cellular systems have only poor metabolic competence and the observed toxicity may vary according to the species, strain and tissue from which the cells originate. In the test programme reported here, tests based on different mammalian cellular systems, on bacterial cultures and specialized animal cells have been compared. Due to the large differences between the various *in-vitro* systems and between the relevant endpoints, three model substances have been tested by the various partners, each using their specific test systems. Each substance could be considered as representative of a class of potential pollutants: inorganic substances (potassium dichromate), solvents (dimethylsulphoxide or DMSO) and organic substances (2′,5′-dimethoxy acetophenone).

Materials and methods

Extraction procedures

The extraction procedures were designed to allow the further toxicity testing in reliable conditions. They were applied to model samples (coded BSP1–8), representing both food-contact materials and other types of paper and board.

Water extraction. Samples were subjected to cold-water extraction employing the procedure documented in EN 645. Extracts were prepared by subjecting 10.0 g of the sample to cold-water extraction at room temperature (20°C) for 24 h. The water extracts were sterile filter before being transferred to a glass bottle.

Ethanol extraction. A total immersion test method was performed according to the pre-standardization method (ENV 1186-15) employing 95% ethanol as the extraction solvent (food simulant). In this procedure, 1 dm² paper sample was cut into small stripes (1 × 3 cm) and placed in a glass cylinder with 50 ml extraction solvent and covered with aluminium foil. The extraction was allowed to proceed without any stirring of the mixture at room temperature (20°C) for 24 h, after which the test pieces were removed from the extraction solution. Ethanol and other volatile components were removed by evaporation and finally extracts were redissolved in ethanol.

Yield of extraction and analysis of extractants. The yields obtained by extracting the paper samples employing these different procedures were determined gravimetrically after removing the extraction solvent and drying the extracts (EN 920). The molecular mass distribution of the ethanol extractants was analysed by size-exclusion chromatography (SEC) and their chemical nature by gas chromatography-mass spectrometry (GC-MS), while the water extracts were characterized employing a procedure involving analytical pyrolysis of aliquots of the water extracts followed by on-line GC-MS of the pyrolysis products thus obtained (the py-GC-MS procedure) as described (Isberg et al. 2002).

Preparation of the positive compound solutions

Three molecules were chosen to cover different types of toxic substances and a wide range of concentrations requested for cytotoxic effects: (1) 2′,5′-dimethoxy-acetophenone (an organic substance, mM range toxicity); (2) potassium dichromate (an inorganic substance, μM range toxicity) and (3) dimethylsulphoxide (DMSO) (a solvent, M range toxicity). Six concentrations of each substance were simultaneously sent by one of the involved laboratories (University of Bourgogne) to the others, together with the dilution instructions, to be tested by each partner using their specific assay. All samples were coded following the requirements needed for a blind ring test.

Cytotoxicity assays with cultured mammalian cells

RNA-synthesis inhibition test. The *in-vitro* RNA synthesis inhibition assay is a short-term test to study the effect of a substance on the viability of cells by measuring the rate of RNA synthesis during 30 min kinetic. The cells are treated with the test chemical and incubated with tritiated uridine, then RNA is precipitated with trichloroacetic acid (TCA), and counted by scintillation liquid method (Fauris et al. 1985). Both HeLa cells and metabolically competent HepG2 cells (Valentin et al. 2001) were used.

Automated test procedure using HepG2 cells. This protocol is previously described by Valentin et al. (2001), with several modifications. The culture medium was EMEM supplemented with 2 mM L-glutamine, 1% non-essential L-amino acids and 10% heat-inactivated foetal bovine serum. Wells of a 96-well tissue culture plate were inoculated with 5×10^4 cells in 0.2 ml culture medium. After a 28-h incubation, the cells were exposed to the test substance for 20 h in EMEM supplemented with 0.5% FBS. For the labelling of RNA, 10 μl tritiated uridine (0.3 μCi/well) were added to each well containing 50 μl cells and medium. Uridine

incorporation was stopped by adding 3% (w/v) sodium dodecyl sulfate (SDS) (30 µl) to each well. After uridine uptake, samples were transferred to a 96-well microplate equipped with GF/C glass filters (Millipore, St Quentin, France). Each filter was wetted with 100 µl 20% TCA just before use. After the application of samples, TCA (100 µl/well) was added again to individual wells to maximize precipitation of nucleic acids and proteins. After 5 min of contact between TCA and cell lysate, the microplate was vacuum filtered using a Manifold system (Millipore) and washed with 200 µl/well ethanol. After 2 h drying in an oven (<60°C), the plate was counted directly in a Top Count microplate reader (Packard, Rungis, France).

Automated test procedure using HeLa cells. The procedure was essentially similar to that applied to HepG2 cells, with the following modifications. The supplementation of the growth medium was 1.5% $NaHCO_3$, 1.0% glutamine, 1% NEEA (Euroclone, Weatherby, UK) and 5% FBS. The cell density at the beginning of a 17-h exposure to the test agent was 6×10^5 ml^{-1}. Labelling was done by adding tritiated uridine (3 µCi) to an aliquot of 700 µl cell suspension. After 6, 12, 18, 24 and 30 min uptakes, 50 µl subsamples were removed into a 96-well microplate and mixed with 30 µl 3% SDS previously pipetted into the wells. The TCA precipitation, filtering, washing with ethanol and drying was done as described for the HepG2 cells. Radioactivity was measured using a Wallac Multiscreen Cassette and a MicroBeta-top count microplate reader (WallacPerkin Elmer Turku, Finland) with 25 µl/well scintillation liquid (Optiphase SuperMix Cocktail, Wallac Perkin Elmer, Turku, Finland).

Cytotoxicity tests with human larynx carcinoma cell line (HEp-2). For tests, the cells were routinely cultivated as a monolayer at 37°C and in 5% CO_2 atmosphere in MEM (Euroclone) medium supplemented with Eagle salts, 5% FBS (Sigma, St Louis, MO, USA), 4 mM glutamine, 0.22% $NaHCO_3$, 200 IU ml^{-1} penicillin and 200 µg ml^{-1} streptomycin. The cells were seeded in 96-well microplates at a density of 9×10^3 cells/well/250 µl medium and treated, when confluent, with the test substance. Alternatively, for the colony-forming ability assay, the cells were seeded in dishes (diameter 35 mm) at a density of 1.5×10^5 cells/dish and, when semi-confluent, treated with different concentrations of the test substance. The following endpoints were used to evaluate the toxicity.

Neutral red uptake (NRU). The assay was performed according to Borenfreund and Puerner (1985). The medium was discarded and the cell monolayer washed three times with phosphate-buffered saline (PBS). The cells were then incubated with the neutral red dye (50 µg ml^{-1} medium) at 37°C for 3 h. After that time, neutral red solution was removed and the wash/fix solution (0.5% formaldehyde, 1% $CaCl_2$) was added into each well and the plate gently shacked for 2 min. After the removal of the wash/fix solution, the extraction solution (50% ethyl alcohol, 1% acetic acid) was added and the plates incubated for 20 min at room temperature. The measurement of absorbance at 540 nm was performed by a microplate reader (Microplate Reader, Model 450, Bio-Rad Laboratories, Milan, Italy).

Total protein content (TPC). The same plates that were used for NRU assay were then washed twice with PBS and the TPC assay was performed after solubilization of the cells in 0.5 M NaOH (Lowry et al. 1951). A mixture of 4% sodium carbonate, 0.04% sodium potassium tartrate and 0.02% cupric sulfate was added to the cells. After 10 min, Folin-Ciocalteu phenol reagent (50% in water) was added and the plates incubated for 30 min at room temperature. The protein concentration in the samples was calculated by the above microplate reader, measuring the absorbance at 630 nm, using a standard curve prepared from bovine standard solution (BSA: 1 mg ml^{-1} in 0.5 N NaOH).

Colony-forming ability (CFA). After a 24- or 48-h treatment, the cells were subcultured at a density of 200 viable cells/dish (diameter 60 mm). After incubation for 1 week at 37°C, the cells were fixed, stained with gentian violet (1% w/v in acetic acid 5% v/v and ethanol 15% v/v) and colonies with more than ten cells were counted (Wilson 1992) by a Colony Counter (Stuart Scientific, UK).

Cytotoxicity tests with the mouse hepatoma cell line (Hepa-1 cells). Cells of the subclone Hepa-1c1c7 of the mouse hepatoma cell line Hepa-1 were grown as a monolayer at 37°C in 5% CO_2 atmosphere in alpha MEM medium (Sigma) supplemented with 1% glutamine, 10% foetal calf serum and 1% penicillin/streptomycin solution. The test was done in 96-well microplates seeded with 200 µl cell suspension (5×10^4 cells ml^{-1}). The cells were exposed to the test substance on the following day, when the culture was about 60% confluent, by replacing the culture medium with a medium

containing either the test substance or the positive control (2,4-dinitrophenol). Untreated cells were used as the negative control. After a 24 h exposure, the cells were washed twice with PBS buffer. Subsequently, 50 µl sodium phosphate buffer (0.05 mM, pH 8.0) were added to each well before freezing the plates for at least 15 min (−70°C). The plates were thawed for 15 min before a further addition of 150 µl sodium phosphate buffer into the wells followed by 50 µl cold 1.08 mM fluorescamine in acetonitrile. The plates were allowed to stand at room temperature for 15 min before being stirred in a microtitration plate shaker for 1 min. The total protein content in each well was measured by the plate-reading spectrofluorometer by using a wavelength of 405/460 nm. BSA standard curves were measured in each bioassay.

Cytotoxicity with the boar spermatozoan motility inhibition test. Extended boar semen, containing 27×10^6 spermatozoa ml^{-1} diluted in commercially available semen extenders obtained from artificial insemination centres was used for the test. The extended semen was exposed to the test substance for 1–4 days at room temperature. Each day the contents of the tubes were mixed by overturning once. Sperm motility was monitored daily and compared with that of the control sample exposed to the solvent or diluent. The minimum sample dilution inhibiting totally the sperm motility was considered as the indicator of the toxicity. In this study the exposure was done in two ways: either according to the standard method by (1) adding 20 µl of each concentration of the test substance to 2 ml semen and subsequently diluting each toxic concentration twofold, until a non-toxic dilution was obtained; or (2) by diluting the test substance in the extended boar semen according to the instructions indicated by University of Bourgogne.

Vibrio fischeri assay as cytotoxicity assay. The *V. fischeri* test was performed as described by Jokinen et al. (2001). This test is a slight modification of the ISO standard 11348-1: 1998(E). Briefly, a stable suspension of *V. fischeri* (DSM 7151) was obtained from a simple 48-h surface culture. The test substances were diluted into water containing 0.4% (w/v) NaCl and added into cuvettes containing the bacterial suspension. Photo-emission was measured automatically using a Bio-Orbit 1251 Luminometry System at 5, 15 and 30 min at 25°C. The concentrations that resulted in a 50% light reduction compared with the blank were regarded as the indicators of the toxicity.

Results

Analysis of samples after extraction

The water extraction typically resulted in relatively high yields of extractables (from 1 to 70.1 mg dm^{-2}), mainly consisting of either complex carbohydrates or lignin-derived oligomeric compounds, depending on the sample (data not shown). Figure 1 shows the total quantity of ethanol extractables along with GC-MS results for some of the preliminary training-phase paper and board samples. As demonstrated, GC-MS analyses showed that except for the extract BSP2, only minor parts of the components in these extract mixtures could be identified by using GC-MS, which detects only low molecular weight substances. The identified organic compounds extracted with 95% ethanol were classified into different chemical classes, i.e. fatty acids, resin acids, sterols and betulinols, sugars, alkanes and alkanols, phthalates and other identified compounds. As shown in Figure 2, the ethanol extract obtained from sample BSP2 was composed mainly of resin acids that probably originated from rosin sizes. The ethanol extracts obtained from the samples BSP7 and BSP8 also contained relatively large portions

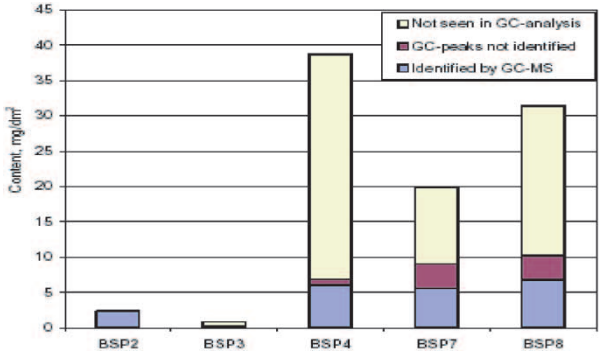

Figure 1. Results obtained by GC-MS analysis of some ethanol extracts of paper and board.

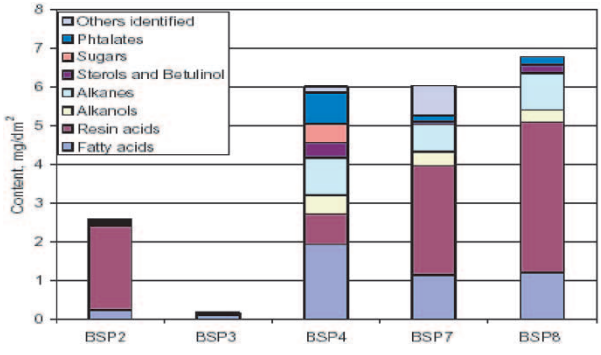

Figure 2. Classes of the substances identified by GC-MS in Figure 1.

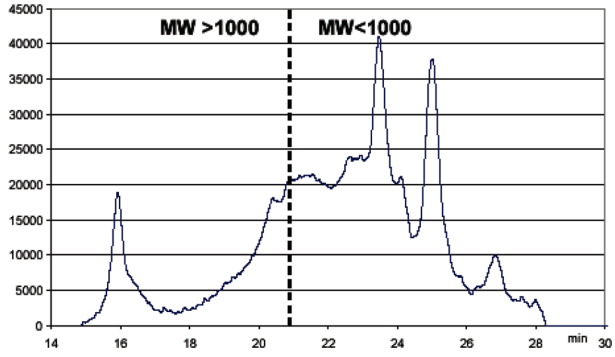

Figure 3. The SEC chromatogram for the 95% ethanol migrate mixture obtained by total immersion extraction of the sample BSP4. The ranges for compounds eluting with retention times corresponding to molar masses above 1000 and below 1000 are separated by a dotted line.

of resin acids according to the data in Figure 2. The molecular weight profile of the ethanol extract of selected samples was determined by size-exclusion chromatography (SEC). The results showed that much of the extract comprised high molecular weight materials (Figure 3). This chromatographic pattern explains why a fraction of the extract was undetected using GC-MS, because such high molecular weight substances are not amenable to GC-MS analysis. High molecular weight substances above 1000 Daltons are generally considered not to be bioavailable and are not usually of toxicological concern.

Analysis of toxic effects of model compounds in different test systems

The relative toxicities (IC_{50}) of the used substances (potassium dichromate, DMSO and 2′,5′-dimethoxy-acetophenone) in the test systems applied are summarized in Table I. 2′,5′-Dimethoxyaceto phenone was toxic in most of the test systems applied, the HEp-2 cell line being a notable exception. In this cell line, the toxicity became evident only at the top doses, and at lower doses there was even some indication of enhanced cell growth. With Hepa-1 cells the toxicity was clearly dose dependent as well as in the RNA-synthesis inhibition tests with both HeLa and HepG2 cells. All tested concentrations inhibited completely the movement of boar spermatozoa, and also suppressed the bioluminescence of *V. fischeri* in a roughly dose-dependent fashion. The EC_{50} values for *V. fischeri*, which were calculated from different test samples, ranged from 0.05 to 0.12 μM. In the case of potassium dichromate, the most sensitive tests system was the *V. fischeri* test, while Hepa-1 cells proved to be the most sensitive mammalian cell test system, the cells being almost totally dead even at the lowest concentrations. Considerable toxicity was observed also with HEp-2 cells, where the NRU was only 35% of the controls at 3.9 μM, the lowest concentration tested. With CFA as an endpoint, the toxicity became apparent only at doses higher than 5.6 μM. In the RNA-synthesis inhibition test with both HeLa and HepG2 cells, a dose-dependent toxic effect was apparent, the EC_{50} values being 5.4 and 8.0 μM, respectively (Table I). In the *V. fischeri* assay, the EC_{50} values ranged from 0.0058 to 0.0085 μM. Potassium dichromate did not inhibit the boar spermatozoan motility at any tested concentration. For DMSO, the consistent phenomenon seen in all the tests applied was the toxicity becoming rather abruptly more prominent at the top doses (0.7 and 0.5 M). Indeed, the HEp-2 cells tolerated the substance well up to the level of 0.5 M, and were actually stimulated at the lower doses. With the Hepa-1 cells the toxicity was somewhat more marked, the EC_{50} being 0.29 M. In the RNA-synthesis inhibition test, HeLa cells were somewhat more sensitive than HepG2 cells the respective EC_{50} values being 0.17 and 0.28 M. In both the boar spermatozoan motility inhibition and *V. fischeri* tests, only the top dose gave a clearly toxic response.

Table I. Relative toxicities of the test substances in the test systems applied.

	EC_{50}						Lowest toxic concentration
	Cytotoxicity assays			RNA-synthesis inhibition assay			
	HEp-2 cells						
Tested compounds	NRU*	CFA	Hepa-1 cells	HeLa cells	HepG2 cells	*Vibrio fischeri* test	Boar spermatozoan motility inhibition assay
Potassium dichromate (μM)	1.9	5.8	<3.9†	5.4	8.0	0.0067‡	Non-toxic at all tested concentrations
DMSO (M)	0.55	0.53	0.29	0.19	0.28	0.02§	0.7
2′,5′-Dimethoxy-acetophenone (mM)	3.1	n.a.	0.69	0.28	1.33	0.00008†	0.8

†Extrapolation of EC_{50} was not possible because of the total cell death at the lowest tested concentration. ‡Mean of the EC_{50} values was obtained from different test concentrations. §Calculated from the test sample with the highest DMSO concentration. n.a., Non applicable.
*The TPC data have been omitted since they coincide with the NRU results.

Discussion

Evaluation of the extraction procedures

Although the extraction procedures and their outcome reported here should be considered as preliminary, they demonstrate — as expected — that the amounts and nature of extractants vary according to the sample and the food stimulant used. It should be recognized that the extraction test using total immersion in 95% ethanol is a severe extraction test and will extract far greater quantities of substances than are likely to migrate to food. Taking into account that the biological tests systems only tolerate up to 2% ethanol, this is however encouraging because it is likely that even these small quantities will contain sufficient material to give a response in the toxicological tests, if the extract contains biologically active material. The application of water extracts is, of course, much more straightforward, as the extracts themselves can be used as the basis of the test media. The results reported here have formed the basis on which the standard operating procedures for the preparation of actual tests samples have been formulated. These will be reported separately.

Performance of the cytotoxicity tests regarding the known toxicity of the model compounds

The test systems applied in this study represent many different types of cells with various metabolic capabilities. The hepatic cell lines (HepG2 and Hepa-1) represent cells that are metabolically able either to activate or detoxify a wide variety of xenobiotics. HEp-2 and HeLa cells are examples of cells with a limited capacity to transform foreign chemicals metabolically, but otherwise possess complete cellular functions. Sperm cells represent highly specialized cells with no major catabolic or biosynthetic activities, and which are highly dependent on the proper mitochondrial functions. Finally *V. fischeri* is a bacterium with a cellular machinery that is highly different from those of the eukaryotic cells. Thus, it can be expected that the test systems based on such divergent cell types give somewhat different responses, and even substances that show toxicity in all of them may have rather different mechanisms of toxicity in each test system. Even with these reservations, the overall agreement between the outcomes of the various tests is rather remarkable, especially in the case with the tests applying cell lines of human or mouse origin. The most notable exception is the relatively low toxicity of 2′,5′-dimethoxyacetophenone in HEp-2 cells, which is all the more surprising taking into account the marked toxicity of this substance in all the other cell lines and even in the boar spermatozoan motility inhibition assay and photobacter test. As there appear to be no previously published reports on the toxicity of this substance, its possible mechanisms of action can, so far, only be speculated. The fact that sperm cells were extremely sensitive to 2′,5′-dimethoxyacetopehnone suggests that it interferes with the proper function of mitochondria and the cellular energy generating machinery. This is further corroborated by the outcome of the *V. fischeri* assay, where the endpoint is the bacterial ATP production. However, there are no known reasons why HEp-2 cells would be more resistant to substances interfering with the respiratory functions than other mammalian tumour cell lines. It is, of course, possible that other toxic mechanisms than impairment of energy metabolism are more important in cultured mammalian cells than in sperm cells, and that HEp-2 cells are somehow more protected against those than other cell lines. Potassium dichromate also showed marked toxicity in all the test systems except for the boar spermatozoan motility inhibition assay. The toxicity of potassium dichromate is based on the presence of hexavalent chromium, which is a strong oxidizing agent, interacting with both small biomolecules (ascorbic acid, glutathione) and macromolecules like proteins and nucleic acids. Potassium dichromate is a carcinogenic and genotoxic agent inducing both the SOS response in *Escherichia coli*, positive response in the Ames *Salmonella* assay and increasing the frequency of micronuclei, chromosomal aberration and mammalian DNA damage both *in vitro* and *in vivo* (Le Curieux et al. 1993; Błasiak and Kowalik 2000; Seoane and Dulout 2001; Fahmy et al. 2002). Moreover, chromium substances are known skin sensitizers (Proctor et al. 1998). At the systemic level, exposure to hexavalent chromium can lead to acute renal failure, probably via the mediation of reactive oxygen species (Barrera et al. 2003). Given the very general toxicological profile of hexavalent chromium, the broad toxicity seen in the present tests is not surprising. The lack of activity in the boar spermatozoan motility assay indicate that mitochondria are not among its primary cellular targets. However, as indicated by the outcome of *V. fischeri* test, potassium dichromate can, directly or indirectly, interfere with bacterial ATP production. The mean EC_{50} value of potassium dichromate obtained in the *V. fischeri* test (0.0067 mM; Table I) was in agreement with the long-term historical EC_{50} of this substance in the performing laboratory (0.007 mM). As an organic solvent, DMSO has an inherent capacity to interact with biological membranes, but it is also known to interfere with many other cellular functions. DMSO is an inducer of differentiation in myeloid and melanoma cell lines (Sawai et al. 1990; Grunt et al. 1991; Miller et al.

1991) and it also induces a reversible G1 arrest in different cell lines at concentrations between 1 and 2% (Forman et al. 1999; Fiore et al. 2002). It can also interact with biotransformation pathways. A protective role of DMSO in relation to nitrofurans has been reported (Ali 1992; Hoogenboom et al 1994). Moreover, it is a very well-known radioprotector (Miyazaki et al. 1990; Watanabe et al. 1990; Sapora et al. 1991) and has been shown to protect against acetaminophen hepatotoxicity (Siegers 1978; El Hage et al. 1983; Jeffery and Haschek 1988). This protective action is suggested to be related to its scavenger property and specifically to its ability to quench hydroxyl radicals (Repine et al. 1979; Jorns et al. 1999). The toxic effects seen in these assays most probably result from the action on the membranes rather than on specific actions on other cellular functions.

Evaluation of the cellular toxicity tests for the test battery to be used in testing the project samples

There is in general a good agreement between the RNA synthesis inhibition test and the different cytotoxicity tests with Hepa-1 or HEp-2 cells, and these test systems apparently form the basis of the final test battery of the cellular toxicity. The boar spermatozoan motility inhibition is a highly specific test that can be used to complement the basic test battery. The usefulness of the *V. fischeri* test is limited because the endpoint is sometimes difficult to interpret (time-dependent alternate stimulation and inhibition of bioluminiscense). Based on the experience gained during the project, the different tests can be graded according to these criteria as presented in Table II.

In conclusion, the tests used to test the actual selected paper and board samples during the latter half of the project include the RNA synthesis inhibition test with HepG2 cell line in combination with cytotoxicity tests with Hepa-1 or HEp-2 cell lines. The considered endpoints and the use of mammalian or human cell lines, with and without biotransformation potency, will give an adequate basis of comparison with cytotoxicity tests already used in the *in-vitro* toxicology field. This basic set of tests will be complemented by the boar spermatozoan motility inhibition test because of its reported sensitivity to certain microbial toxins like cereulide.

The suggested combination of tests will detect toxic samples at a high probability. In cases where a positive result is obtained from a single test only, the interpretation will be crucial in order to eliminate the false-positives, but would be helped by the use of different endpoints and different cell lines. The final criteria for the interpretation of the results are to be developed in Module III during years 3 and 4. In short, the results of this study demonstrate that different short-term assays can give qualitatively similar results with the same test substance, particularly when the toxic endpoints are not very specialized. Tests based on a very defined cellular target,

Table II. Grading of the cellular toxicity tests according to the acceptance criteria.

Test	Relative sensitivity	Consistency	Discriminatory power
Vibrio fischeri test	Average; toxic compounds recognized sometimes at rather high concentrations only	Good; good historical record of consistency	Poor; endpoint sometimes difficult to interpret
Boar spermatozoan motility inhibition test	Good; very sensitive to cereulide and related compounds; the sensitivity to other chemicals, when recognized, comparable with that of the actual cytotoxicity tests	Good; test has been successfully adapted by a new partner during the BIOSAFEPAPER project	Average; some toxic compounds not recognized by the test
RNA-synthesis inhibition test (HepG2 cell line)	Good; positive control samples detected even at the lowest concentrations	Average; good historical record of consistency; the test has been successfully adapted by a new partner during the BIOSAFEPAPER project, when established, gives a consistent response; set up in a new laboratory needs real expertise	Good; all positive control samples detected; occasionally the only test that gives a response with unknown samples; the possibility of false-positives should be evaluated
Cytotoxicity test (Hepa-1 cell line)	Good; positive control samples detected even at the lowest concentrations	Good; good historical record of consistency	Good; all positive control samples detected
Cytotoxicity test (HEp-2 cell line)	Good; positive control samples detected at low concentrations	Good; good historical record of consistency	Good; most positive control samples detected

The table also reflects, in addition to the results reported here, the cumulative experience gained during the subsequent course of the project with other chemicals and samples tested.

such as the boar spermatozoan motility test used here, may give useful hints about the possible toxicological mechanism of a given substance, but may miss some quite important toxic agents. Consequently, for routine testing, a useful test battery should contain assays able to detect a broad range of different toxic effects, complemented with tests with special endpoints giving an indication of the cellular or metabolic functions affected by the tested agents. It is emphasized that this present regime does not anticipate the final tests that should be included in the recommended test battery, but rather the need to rationalize the testing procedure for the project samples.

Further activities

Since the mid-term evaluation the cytotoxicity test battery outlined above is being used to test both the model extracts of the BSP series, certain wood- and paper-and-board-associated chemicals and contaminants, and especially the actual, paper and board samples selected by the industry. This work is ongoing, and the outcome of the testing will be published after the completion of the studies and thorough analysis of the results. In addition to the cytoxicity assays reported here, also the genotoxicity test battery applicable to paper and board samples, which is one of the deliverables of the project, has been finalized (manuscript in preparation).

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References

Acosta D, Anufuro DC, Smith MT. 1980. Cytotoxicity of acetaminophen and papaverine in primary cultures of rat hepatocytes. Toxicology and Applied Pharmacology 53:306–313.

Ali BH. 1992. Effect of furazolidone on tissue sulphydryl groups, ascorbic acid and lipid peroxide levels in the rat, and the influence of dimethylsulphoxide thereon. Pharmacological Research 25:247–254.

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. Applied and Environmental Microbiology 64:1338–1343.

Anon. 1995. ICCVAM: US ad hoc Interagency Coordinating Committee on validation of Alternative Methods. ATLA 23:149.

Balls M, Blaauboer B, Brusick D, Frazier J, Lamb D, Pemberton M, Reinhardt C, Robertfroid M, Rosenkranz H, Schmid B, Spielmann H, Stammati A, Walum E. 1990. Report and recommendations of the CAAT/ERGATT workshop on the validation of toxicity test procedures. ATLA 18:313–337.

Balls M, Blaauboer B, Fentem JH, Bruner L, Combes RD, Ekwall B, Fielder RJ, Guillouzo A, Lewis RW, Lovell DP, Reinhardt CA, Repetto G, Sladowski D, Spielmann H, Zucco F. 1995. Practical aspects of the validation of toxicity test procedures. ATLA 23:129–147.

Balls M, Bridges JW. 1984. The FRAME research programme on in vitro cytotoxicology. In: Goldberg AM (editor). Acute Toxicology Testing: Alternative Approaches, Mary Ann Liebert, Inc., New York, pp. 63–79.

Balls M, Fentem JH. 1997. Progress toward the validation of alternative tests. ATLA 25:33–43.

Barrera D, Maldonado PD, Medina-Campos ON, Hernandez-Pando R, Ibarra-Rubio ME, Pedraza-Chaverri J. 2003. Protective effect of $SnCl_2$ on $K_2Cr_2O_7$-induced nephrotoxicity in rats: the indispensability of HO-1 preinduction and lack of association with some antioxidant enzymes. Life Science 73:3027–3041.

Błasiak J, Kowalik A. 2000. A comparison of the in vitro genotoxicity of tri- and hexavalent chromium. Mutation Research 469:135–145.

Borenfreund E, Puerner JA. 1984. A simple quantitative procedure using monolayer cultures for cytotoxicity assays. Journal of Tissue Cultures Methods 9:7–12.

Borenfreund E, Puerner JA. 1985. Toxicity determined in vitro by morphological alterations and neutral red absorption. Toxicology Letters 24:119–124.

Broadhead CL, Combes RD. 2001. The current status for food additives toxicity testing and the potential for application of the Three Rs. ATLA 29:471–485.

Clemedson C, Barile FA, Chesne C, Cottin M, Curren R, Eckwall B, Ferro M, Gomez-Lechon MJ, Imai K, Janus J, Kemp RB, Kerszman G, Kjellstrand P, Lavrijsen K, Logemann P, McFarlane-Abdulla E, Roguet R, Segner H, Thuvander A, Walum E. 2000. MEIC evaluation of acute systemic toxicity. Part VII. Prediction of human toxicity by results from testing of the first 30 references chemicals with 27 further in vitro assays. ATLA 28:159–200.

El Hage AN, Herman EH, Ferrans VJ. 1983. Examination of the protective effect of ICRF-187 and dimethyl sulphoxide against acetaminophen-induced hepatotoxicity in Syrian golden hamsters. Toxicology 28:295–303.

Fahmy MA, Shoman HM, Hassan EE. 2002. The protective role of thiols and soybean seeds against the genotoxicity induced by potassium dichromate in mice. Mutation Research 517:1–12.

Fauris C, Danglot C, Vilagines R. 1985. Rapidity of RNA synthesis in human cells. Water Research 19:677–684.

Fiore M, Zanier R, Degrassi F. 2002. Reversibile G(1) arrest by dimethyl sulfoxide as a new method to synchronize Chinese hamster cells. Mutagenesis 17:419–424.

Forman S, Káš J, Fini F, Steinberg M, Ruml T. 1999. The effect of different solvents on the ATP/ADP content and growth properties of HeLa cells. Journal of Biochemical and Molecular Toxicology 13:11–15.

Grunt TW, Somay C, Pavelka M, Ellinger A, Dittrich E, Dittrich C. 1991. The effects of dimethyl sulfoxide and retinoic acid on the cell growth and the phenotype of ovarian cancer cells. Journal of Cell Science 100:657–666.

Holden HT, Lichter W, Sigel M. 1973. Quantitative methods for measuring the cell growth and death. In: Kruse P, Patterson MK, editors. Tissue culture: methods and applications. New York: Academic Press. p 408.

Hoogenboom LAP, Polman THG, Lommen A, Huveneers MBM, Van Rhijn J. 1994. Biotransformation of furaltadone by pig hepatocytes and Salmonella typhimurium TA 100 bacteria and the formation of protein-bound metabolites. Xenobiotica 24:713–727.

Isberg K, Jansson, MB, Hardell H.-L, Dahlman O. 2002. Extractable components in paper for food contact-characterization of high molecular weight mass material. Nordic Pulp and Paper Research Journal 17:172–178.

Jeffery EH, Haschek WM. 1988. Protection by dimethylsulfoxide against acetaminophen-induced hepatic, but not respiratory toxicity in mouse. Toxicology and Applied Pharmacology 93:452–461.

Jokinen K, Savolainen M, Söderhjelm L. 2001. Photobacterium test for evaluation of toxicity of fibrous products. Paperi ja Puu 83:332–335.

Jorns A, Tiedge M, Lenzen S, Munday R. 1999. Effect of superoxide dismutase, catalase, chelating agents and free radicals scavengers on the toxicity of alloxan to isolated pancreatic islets in vitro. Free Radicals in Biology and Medicine 26:1300–1304.

Korzeniewski C, Callewaert DM. 1983. An enzyme release assay for natural cytotoxicity. Journal of Immunological Methods 64:313–320.

Le Curieux F, Marzin D, Erb F. 1993. Comparison of three short-term assays: results on seven chemicals. Potential contribution to the control of water genotoxicity. Mutation Research 319:223–236.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193:265–275.

Miller DR, Allison DP, Rorvik MC, Slaga TJ. 1991. Inhibited morphological terminal differentiation and enhanced proliferation of cultured mouse epidermal cells at different concentrations of dimethyl sulphoxide. Cell Proliferation 24:191–210.

Miyazaki T, Hayakawa Y, Suzuki K, Watanabe R. 1990. Radioprotective effects on dimethyl sulfoxide in golden hamster embryo cells exposed to rays at 77 k. I. Radical formation as studied by electron spin resonance. Radiation Research 124:66–72.

Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. Journal of Immunological Methods 65:55–63.

OECD. 1996. Harmonization of validation and acceptance criteria for alternative toxicological test methods. Workshop, Solna, Stockholm, 22–24 January.

Phillips HJ. 1973. Dye exclusion tests for cell viability. In: Kruse Jr, PF & Patterson Jr, MK, editors. Tissue culture: methods and applications. New York: Academic Press. p 406.

Proctor DM, Fredrick MM, Scott PK, Paustenbach DJ, Finley BL. 1998. The prevalence of chromium allergy in the United States and its implications for setting soil cleanup: A cost-effectiveness case study. Regulatory Toxicology and Pharmacology 28:27–37.

Rahn CA, Bombick DW, Doolittle DJ. 1991. Assessment of mitochondrial membrane potential as an indicator of cytotoxicity. Fundamental and Applied Toxicology 16:435–448.

Repine J, Eaton J, Anders M, Hoidal J, Fox R. 1979. Generation of hydroxyl radical by enzymes, chemicals and human phagocytes in vitro. Journal of Clinical Investigations 64:1642–1651.

Sapora O, Barone F, Belli M, Maggi A, Quintiliani M, Tabacchini MA. 1991. Relationships between cell killing, mutation induction and DNA damage in X-irradiated V79 cells: the influence of oxygen and DMSO. International Journal of Radiation Biology 60:467–482.

Sawai M, Takase K, Teraoka H, Tsukada K. 1990. Reversible G1 arrest in the cell cycle of human lymphoid cell line by dimethyl sulfoxide. Experimental Cell Research 187:4–10.

Seoane AI, Dulout FN. 2001. Genotoxic ability of cadmium, chromium and nickel salts studied by kinetochore staining in the cytokinesis-blocked micronucleus assay. Mutation Research 490:99–106.

Shopsis C, Sathe S. 1984. Uridine uptake inhibition as a cytotoxicity test: correlations with the draize test. Toxicology 29:195–206.

Siegers CP. 1978. Antidotal effects of dimethyl sulphoxide against paracetamol-, bromobenzene- and thioacetamide-induced hepato-toxicity. Journal of Pharmacy and Pharmacology 30:375–377.

Thelestam M, Molby R. 1976. Cytotoxic effects on the plasma membrane of human diploid fibroblasts, a comparative study of leakage tests. Medical Biology 18:54–65.

Valentin I, Philippe M, Lhuguenot JC, Chagnon MC. 2001. Uridine uptake inhibition as a cytotoxicity test for a human hepatoma cell line (HepG2 cells): Comparisons with the neutral red assay. Toxicology 158:127–139.

Watanabe M, Suzuki M, Suzuki K, Hayakawa Y, Miyazaki T. 1990. Radioprotective effects of dimethyl sulfoxide in golden hamster embryo cells exposed to rays at 77. II. Protection from lethal, chromosomal and DNA damage. Radiation Research 124:73–78.

Wilson AP. 1992. Cytotoxicity and viability assay. In: Freshney RI, editor. Animal cell culture — a practical approach series. Oxford: IRL. p 263.