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Application of bioassays for safety assessment of paper and board for food contact

Final report BIOSAFEPAPER (QLK1-CT-2001-00930)

Application of Bioassays for Safety Assessment of Paper and Board for Food Contact

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ABBREVIATIONS

BNC	binucleated cells
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
CFA	colony forming ability
CO_2	carbon dioxide
Cr VI	chromium VI
DNA	deoxyribonucleic acids
EC_{50}	effective concentration at which 50 % of maximum effect is obtained
EDTA	ethylenediaminetetraacetic acid
EMEM	Eagle Minimum Essential Medium for cell cultures
FBS	foetal bovine serum
HeLa cells	human cervical carcinoma cell line
Hepa-1 cells	mouse hepatoma cell line
HepG2 cells	human hepatocellular carcinoma cell line
LMP	low melting point
MEM	Minimum Essential Medium for cell cultures
MN	micronucleus
NaOH	sodium hydroxide
NEAA	non-essential amino acid supplement for cell culture media
NMP	normal melting point
NRU	neutral red uptake
PBS	phosphate buffered saline
RNA	ribonucleic acids
S-9	microsomal preparation from rat liver
SCG/Comet	assay for DNA mutations
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TPC	total protein content

1. CONTRIBUTORS

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2. SUMMARY NOT CONFIDENTIAL

Objectives:

The aim of Biosafepaper project was the development and intercalibration of a short-term test battery for safety assessment of food contact paper and board. *In vitro* toxicity tests developed and applied previously in various national projects formed the background of the project, which was to be carried out at a pre-normative level in order to harmonize these tests. Further, the aim was to adapt these tests for safety assessment of paper and board used for food contact. The final aim is to provide a basis for scientifically sound recommendations for a harmonized risk evaluation and product testing. The results are disseminated to industry, legislators and consumers and will form a common standard for safety evaluation.

Results and Milestones:

Expected results

Decision tree based approach to toxicity testing

Basis for scientifically sound recommendations for harmonised risk evaluation

Improved understanding between stakeholders on risk

Milestones

Development and intercalibration of toxicity tests

Validation of new tests for sublethal cellular toxicity between partners

Recommendation of a test battery

Standard operating procedure (SOP) for extraction protocol

Internal and extended audience workshops on results

Achievements:

The project consists of three modules: Module I (Toxicological testing), Module II (Extraction procedures) and Module III (Implementation in risk assessment). Since the main task of Module II was to provide test materials to Module I, the modules are here presented starting with Module II, followed by Modules I and III.

Module II

The main purpose of the Module II (Extraction procedures) was to provide training materials for the eventual use of the teams in the Module I. These materials were; test extracts of paper and board and individual test substances (including natural constituents of paper and board as well as man-made contaminants). These materials were required by the toxicology teams to test, characterise, and then validate the capabilities and the limitations of the various *in vitro* assays that they propose. The chemical characterization of the extracts was also part of the activities of this module.

Working closely with the industrial partners, 20 representative P/B samples were selected to be tested in Module I. The 20 samples cover a wide range of applications and they include some samples not intended for food contact use. In this way, the in vitro toxicity testing of extracts/migrates from these 20 sample types was concordant with the Pre-validation Phase of ECVAM (European Centre for the Validation of Alternative Methods) work in which the scope of any new, non-animal, methodology should be evaluated using samples representative of very different products.

The most important outcome of Module II was the standard operating procedure SOP for preparing extracts intended for toxicity testing. The extractants selected were water for materials intended for wet foods, ethanol as a standard simulant for fatty foods, and Tenax for dry foods (ethanol is the final solvent also with Tenax extracts). While the standard CEN water extraction procedures designed for chemical migration studies could be applied for materials intended for wet foods, the procedures had to be modified for paper and board intended for fatty or dry foods. The reason for this is the fact that, while water extracts can be applied in biological tests as undiluted (indeed, the test media can be reconstituted using the extract as the solvent), the tolerance of the biological systems to ethanol is limited to appr. 2 %. Consequently with both ethanol and Tenax extracts the test material-extractant ratio was maximized, and the ethanol extracts were further concentrated. This way the concentrations of ethanol dissolved extractables at the applied concentration (max 2 %) in the biological tests were made comparable to those obtained with water extracts (applied at 80 - 90 % concentration).

Module I

In the Module I (Toxicological testing) the main tasks were:

1. The standardisation of the toxicological tests applied by different scientific partners. The tests considered in the beginning of the project included tests measuring sublethal cellular events, such as the RNA-synthesis inhibition test, the boar spermatozooan motility test and the bacterial bioluminescence test as well as more traditional cytotoxicity tests with the cell death as an endpoint. Also the suitability of various genotoxicity assays measuring both point mutations and DNA-damage was assessed.
2. The application of these tests to model chemicals representing either wood associated substances or suspected chemical contaminants of paper and board
3. To use tests selected on the basis of their performance in 1. and 2. to screen extracts of a selection of paper and board samples selected by industry. The extracts were prepared according to the standard operating procedure (SOP) developed in Module II.

The main outcome of Module I was the selection of a combination of tests that could be both applied to paper and board extracts and form the basis of **the decision -tree approach** (see Module III). **When applied to actual paper and board extracts these tests correctly identified samples that intentionally represented non-food grade materials (see Module II).** The tests were performed blind by the laboratories not knowing the identity of the samples.

Module III

The main tasks of Module III (Implementation in risk assessment) were to establish an ad hoc group involving external assessors on risk assessment and to ensure that the project is updated on the progress concerning toxicological methodologies through literature surveys. The ad hoc group was very active providing useful recommendations and advise during the course of the project.

The main outcomes regarding implementation in risk assessment have been a decision tree-based approach for hazard identification based on the selected in vitro tests, and the application of the concept of Correction Factors (CF) in the interpretation of the results. CFs take into account the necessity to use as rigorous extraction procedures as possible to reach the detection limits of the biological assays and translates the results to the realistic exposure levels that a consumer might meet. CFs are obtained by taking into account the intended end use, what is known about the actual migration levels and the nature of the food contact. In practice they give an indication of the dilution that should be

applied to the extract to get an estimate of the realistic level of exposure. The CF concept was introduced during the project, and obviously needs further refinement in order to be fully applicable.

Module III was also in charge of communication and dissemination, the main effort has been in creating channels for both internal and external communication.

The meetings arranged for the different modules have been fruitful as forums for scientific discussions and also served the very important purpose of bringing the groups together and building up networks and trust between the members. The external communication included poster presentations at scientific conferences, presentations in workshops arranged by the packaging industry, creation and regular updating of a web site and production of project leaflets.

Benefits and Beneficiaries:

This project represents a prenormative research effort to address consumer and regulatory concerns by a decision-tree approach using short-term tests with end points relevant to the genuine risk involved in the end use. The successful outcome will enable paper and board producers to promote their products on the basis of sound, transparent, scientific criteria making them highly adapted to to-day's sophisticated, safety conscious food market.

The project has been necessary in order to launch harmonisation of risk assessment for food contact paper and board at European level. The development of European regulations will stabilise European markets and reduce the risk of regulations being imposed by non-European actors.

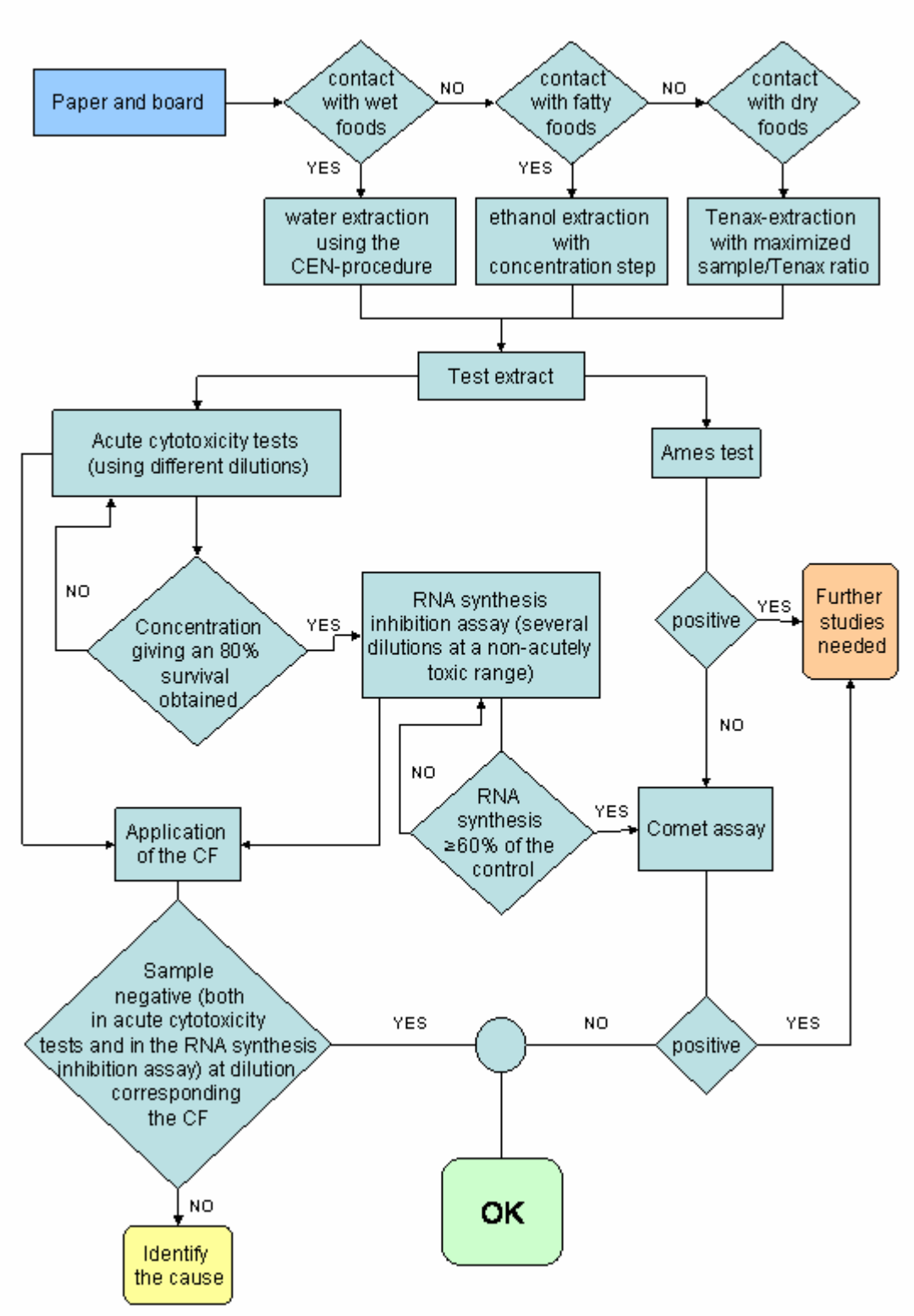
Through this project it has been possible to establish cooperation between the partners and to create a network between the scientific and industrial communities. Especially from the industrial point of view the project meetings form platforms for discussions on topics of utmost importance.

Conclusions

The main expected outcomes of the project were:

1. A decision-tree based approach to safety evaluation
2. The standard operating procedure (SOP) for extraction protocols
3. Basis of scientifically sound recommendations for harmonized risk evaluation

These have been all achieved. Of particular importance is the introduction of the concept of Correction Factors in no 3. The realization of the improved understanding between stakeholders on risk, another expected outcome of the project, has been achieved between the scientific and industrial project partners. The spread and wider acceptance of this understanding among other actors (consumer organizations, regulatory and expert bodies) depends on the successful dissemination of the results. This effort continues by the different partners even after the ending of the project.



The decision tree based test battery for hazard identification based on the experience gained during the project

3. INTRODUCTION

Product safety is the prime concern of the European paper and board manufacturers. For chemicals that are intentionally added during the manufacturing process the safety can be evaluated using approaches similar to those described for plastics in the corresponding guidelines. However, while migration tests and chemical analysis of known harmful compounds are available, they cannot be applied to a product with an incompletely defined chemical composition, such as paper. Instead, hazard analysis should be applied to the manufacturing process and special control measures used for the end products. Currently, this latter need can only be satisfied by using suitable toxicological tests combined with relevant migration conditions to give an indication of the potential risks involved. Such traditional tests for acute or chronic toxicity are time consuming, expensive, and associated with ethical concerns (animal experiments). Moreover, they are very difficult to apply to products like paper and board, and totally unsuitable for assessment of products and processes, for example in the hazard analysis context. Consequently, various short term tests using bacterial cultures or cultured mammalian cell lines have been used for quick testing of various complex materials such as foodstuffs, chemicals and environmental samples. Different short term tests have, however, different endpoints, and contradictory results from similar types of samples tested with different systems are common. A disadvantage of various traditional short term tests for cellular toxicity is that sublethal effects, which might be the only toxic indications when small quantities of unknown substances or complex mixtures are tested, are easily overlooked.

In summary, there was a need for **a prenormative project** in order to establish a set of tests that would:

- Be easily adaptable to food contact paper-based materials
- Have endpoints relevant to actual consumer safety, including sublethal cellular events.
- Be based on samples representing actual migration conditions from food contact paper and board into different foodstuffs
- Be quick, cost effective and easy to establish and validate
- Be applicable to hazard analysis procedures designed for production processes
- Form a basis for scientifically sound recommendations for a harmonised system of risk evaluation and product testing

This BIOSAFEPAPER project has been designed to fulfil the criteria listed above. The **objectives** of the project are the development of:

- **a test battery,** with a decision-tree approach with end points relevant to consumer safety, will be developed. Novel test systems measuring sublethal effects on mammalian cells, cross–validated by the project partners will be included;
- **extraction procedures** applicable to paper and board with solvent/adsorbent systems simulating different types of foodstuffs and developed from end use applications;
- **risk assessment procedures** based on the scientific evaluation of the results;
- **dissemination** of the results to consumers, legislators and industry to form a basis for regulatory activities.

Expected **achievements** from this project are:

- tools to assess the safety of food contact paper and board products;
- harmonisation of testing procedures applicable both to regulatory purposes and for the hazard analyses approach in the industry;
- the increased confidence of consumers in the will and ability of European industries to continue providing safe food contact materials;
- improved industry competitiveness due to the development of robust methods of assuring product safety and fitness for use.

4. MATERIALS AND METHODS

4.1. General approach

The project consists of three modules, and their relationships and interactions with each other are indicated in the Pert diagram (Figure 1).

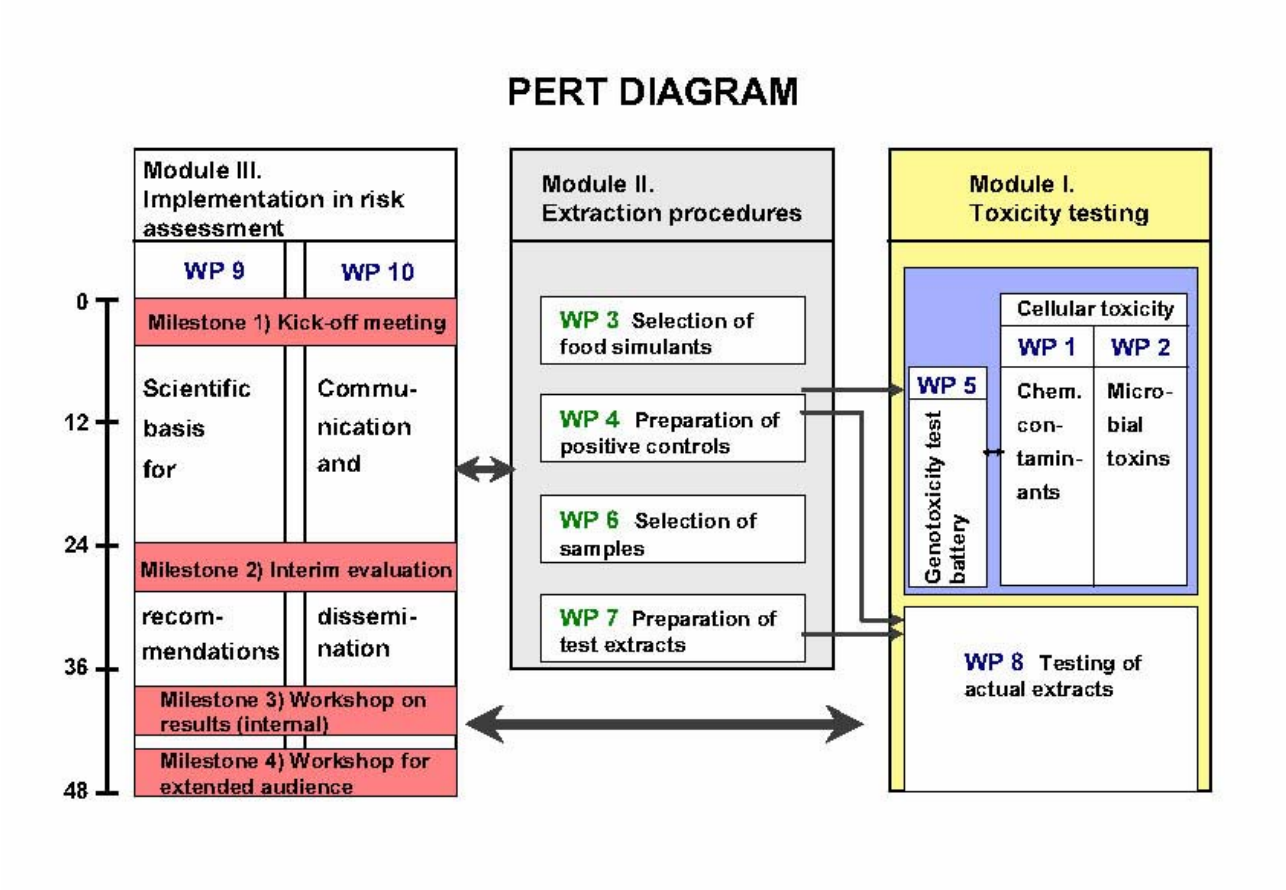


Figure1. Pert diagram.

Module 1 (toxicity testing)

The different test systems available for the project are outlined in Figure 2. The work involved adapting the agreed toxicological tests in different participating laboratories. This applied particularly to newly developed tests for sublethal toxicity (RNA-synthesis inhibition test, boar spermatozoan motility inhibition test). These measures were carried out to standardise the procedures and using historical positive control samples. These samples were also tested in classical cytotoxicity tests, which provide an external standard for specificity and sensitivity.

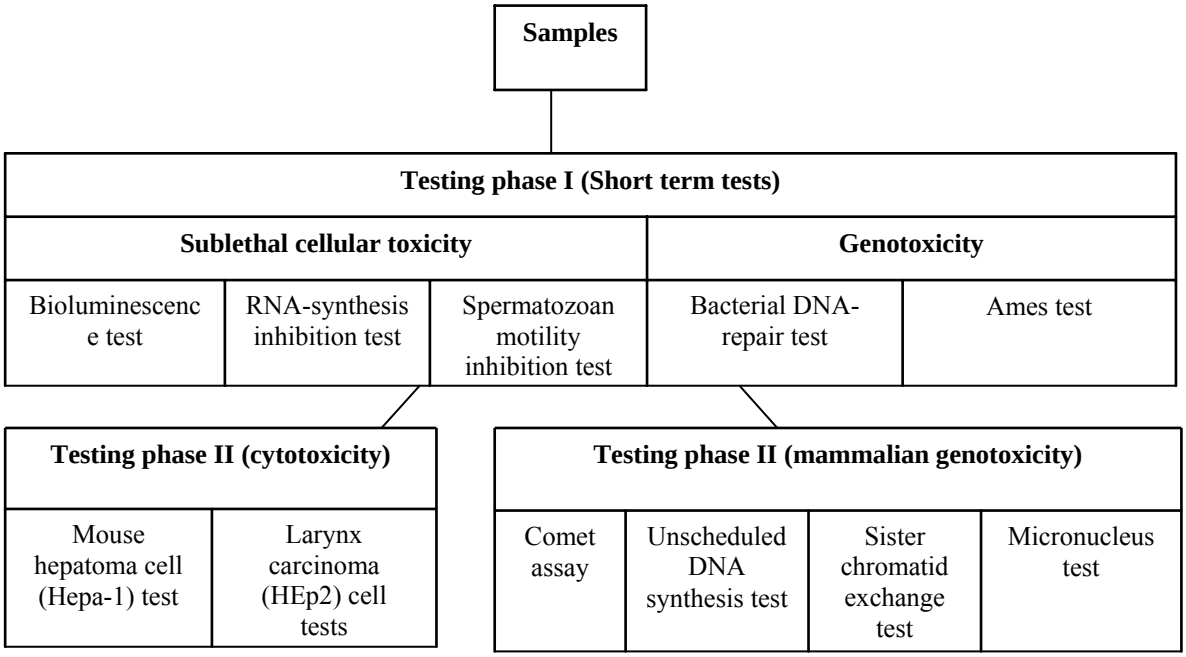


Figure 2. The battery of short term tests available for the project.

Also the more established genotoxicity tests were carried out by at least two partners. After the tests had been established, positive control samples were tested. These were obtained from Module II (see below). On the bases of the responses the final test battery for measuring the toxicities of actual real life paper and board extracts was selected and applied.

Module 2 (extraction procedures)

In this module realistic extraction procedures were developed for the toxicity testing of paper and board. This was done by using paper and board extracts spiked with known toxic substances and applying different extraction strategies taking into account the requirements of different food matrices, and the compatibility with the biological test systems.

Module 3 (implementation and risk assessment)

In this module the results of the project were internally continuously evaluated against the criteria defined both for the toxicity testing and extraction procedures in order to ensure the robustness and final applicability of the approach. On the basis of the experiences gained, the test protocols were outlined in close cooperation between the Modules I and II. The results were communicated to different stakeholders (industry, authorities and consumers) in order to

ensure the general practicability and acceptance of the outcome. The final result was a set of recommendations that can serve as a bases of an European regulatory framework for food contact paper and board.

4.2. Test materials

4.2.1. Model chemicals

Model compounds used in the comparison of the performance of the cytotoxicity test systems
Three molecules were chosen in order to cover different types of toxic substances and a wide range of concentrations requested for cytotoxic effects:

- **2',5'-dimethoxy-acetophenone** (an organic substance, mM range toxicity);
- **potassium dichromate** (an inorganic substance, µM range toxicity) and
- **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.

In addition, **cereulide**, the emetic toxin of *Bacillus cereus,* was chosen to represent toxins of biological origin. The strain F4810/72 used is the best known and widely used emetic toxin (cereulide) producing strain of *B. cereus* (Turnbull et al., 1979, Andersson et al., 1998a, 2004). The cereulide non-producing strain used was *B. cereus* NS61, isolated from a spruce tree (Hallaksela et al., 1991) and confirmed by bioassay and by LC-MS not to produce cereulide (Andersson et al., 2004) with a detection limit 0.9 ng mg^{-1} cells fresh wt.
The *B. cereus* extracts were prepared from 3 to 4 grams (fresh wt) of biomass grown on tryptic soy agar plates for 24 h at 28°C plates. The bacterial biomass was extracted with methanol as described in Andersson et al. (1998a). The extracts were heated for 10 min at 100°C and the cereulide concentration determined by LC ion trap MS assay based on the cereulide specific mass ions with m/z of 1175 (Na^+ adduct), 1192 (K^+ adduct), 1171 (NH_4^+ adduct) and 1154 (H^+) and calibrated with valinomycin as described by Jääskeläinen et al.

(2003 a). The extracts contained 38.4 mg (strain F4810/72) and 38.6 mg (strain NS 61) of dry substance and 90µg and <0.01 µg of cereulide / ml of extract, respectively.

Substances representing natural compounds or potential contaminants

The responses of the test systems towards different compounds that represent either natural substances present in wood or potential contaminants in final paper and board products were studied. The selected compounds and substances are listed in Table 1.

Table 1. The test substances

Model compounds or wood related substances	**Relevance to paper and board**	**Solvent in the test systems**	**Source**
Dihydroabietic acid	Natural resin acid	ethanol	Provided by STFI
Spruce wood ethanol extract	Natural components of raw materials	ethanol	STFI (the content of organic material 1. 2 g l^{-1})
Spruce wood hot water extract	Natural components of raw material	water	STFI (prepared using 50 ºC water to extract spruce wood meal for 4 h; the final extract contains appr. 7 g l^{-1} organic material)
Acrylamide	Contaminant possibly arising from polyacrylamides used during paper making	ethanol water	Sigma
2,4 - Diaminotoluene	Environmental pollutant	DMSO	Aldrich, Merck
Phthalate mixture (DiBP, DBP and DEHP in a ratio of 4:1:1)	Possible contaminants in recycled paper products	DMSO	Prepared by CSL-MAFF, the pure chemicals from Aldrich
Di-isopropylnaphthalenes (DiPNs)	Possible contaminant in recycled papers	DMSO	Fischer
Disulpho-stilbene (Blancophor disulphone)	Optical brighteners	water	Blancophors (Bayer)
Tetrasulpho-stilbene (Blancophor tetrasulphone)	Optical brighteners	water	Blancophors (Bayer)

4.2.2. Board and paper samples

Model samples (BSP series)

In order to evaluate the standard extraction procedures and to get an idea of the chemical nature of typical paper and board extracts nine different model materials were chosen (Table 2).

Table 2. Description of paper and board samples employed for preparation of migrant mixtures simulating different types of food contact applications.

Code	**Type**	**Pulp**	**Recycled**	**Grammage** (g/m^2)	**Thickness** (µm)	**Density** (kg/m^3)
BSP1	Filter paper	Virgin fibers	0%	13.3	56	237
BSP2	MG-Paper	Bleached kraft	0%	71,5	82	868
BSP3	Greaseproof paper	Bleached sulfite	0%	41	50	820
BSP4	Corrugated board	Test liner-fluting - test liner	Yes	727	4181	174
BSP5	Test liner	Recycled	Yes	240	385	625
BSP6	Fluting	Semi chemical	0%	170	274	620
BSP7	Chipboard	Recycled	100%	406	576	706
BSP8	White lined chipboard	Recycled	90%	497	719	691
BSP9	SBS	Bleached kraft	0%	199	259	768

Nominated samples for the project (NSP serie)

The actual project samples were selected by the industry, and assigned codes by CSL (Table 3).

The extracts were prepared according to the intended end use either in STFI (water extracts) or CSL (ethanol and Tenax extracts). The samples were then sent to different laboratories for testing. The testing laboratories only knew the NSP code and the final solvent used.

Table 3. Samples supplied by industrial sponsors, showing chosen simulants in each case

Code	Description	Type of simulated food contact	Grammage (g/m^2)	Recycled content	Time/temperature conditions of intended use	Simulant (g sample / L simulant or g Tenax / dm^2 sample)	Time / temperature conditions of exposure/extraction
NSP1	Solid Board	dry	665	100%	1 week at 4°C	Tenax (1 g/dm^2)	24 hours at room temperature
NSP2	Board GD2 (WLC)	dry	300	84%	months at ambient temperature	Tenax (1 g/dm^2)	5 days at 50°C
NSP3	Board GD2 high newsprint	dry	300	100%	months at ambient temperature	Tenax (1 g/dm^2)	5 days at 50°C
NSP4	Board GD3 (WLC)	fat	300	100%	not relevant non food grade	95% Ethanol (100 g/L)	24 hours at room temperature
NSP5	Board GD *grease resistance treated*	fat	500	90%	months at ambient temperature	95% Ethanol (100 g/L)	24 hours at room temperature
NSP6	Board GD *water resistance treated*	wet	300	98%	minutes at ambient *	Water (40 g/L)	CEN-cold
NSP7	Folding boxboard *wet strengthened*	wet	285	0%	1 week at 8°C	Water (40 g/L)	CEN-cold
NSP8	White top kraftliner	fat	140	0%	minutes at hot *	95% Ethanol (100 g/L)	24 hr at RT
NSP9	Unbleached liner recycled, *surface sized*	dry	170	100%	weeks at ambient temperature	Tenax (1 g/dm^2)	5 days at 50°C
NSP10	Bleached liner recycled	dry	140	100%	a few weeks at ambient temperature	Tenax (1 g/dm^2)	10 days at 20°C
NSP11	Uncoated SBS	wet	210	0%	minutes hot	Water (40 g/L)	CEN-hot
NSP12	Recycled fluting dry food	dry	105	100%	months at ambient temperature (indirect contact)	Tenax (1 g/dm^2)	5 days at 50°C

Code	Description	Type of simulated food contact	Grammage (g/m^2)	Recycled content	Time/temperature conditions of intended use	Simulant (g sample / L simulant or g Tenax / dm^2 sample)	Time / temperature conditions of exposure/extraction
NSP13	Corrugated board (virgin), p*igment coated*	fat	666	0% (inner liner is 20% recycled)	months at frozen *	95% Ethanol (100 g/L)	24 hours at room temperature
NSP14	Corrugated board recycled	dry	500	100%	up to 4 weeks frozen *	Tenax (1 g/dm^2)	10 days at 20°C
NSP15	Unbleached sulphate Kraft paper virgin	dry	70	0%	months at ambient temperature	Tenax (1 g/dm^2)	5 days at 50°C
NSP16	Paper based on deinked fibre	dry	40	100%	1 day (maximum) at ambient temperature	Tenax (1 g/dm^2)	24 hours at room temperature
NSP17	Folding boxboard; *pigment-coated*	dry	255	0%	months at ambient temperature *	Tenax (1 g/dm^2)	5 days at 50°C
NSP18	Solid bleached board, *pigment-coated*	wet	240	0%	months frozen	Water (40 g/L)	CEN-cold
NSP19	CNB	wet	300	0%	months at all temperatures	Water (40 g/L)	CEN-cold
NSP20	Hard sized paper	wet	30	80%	1 day (maximum) at ambient temperature	Water (40 g/L)	CEN-cold

Note. Nearly all producers reported internal sizing treatments

* indicates other uses or temperature also given

4.3. Extraction procedures

There were 3 guiding principles for preparing the extracts of the paper/board samples for the bioassay procedures:

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

How these criteria were met is described in Table 4.

Table 3. provides a description of the samples tested and the rational for the selection of the extraction tests conducted. Of the 20 NSP samples described in Table 3., six (6) samples were assigned for extraction using water, six (6) were assigned for extraction using ethanol, and ten (10) were assigned for extraction using Tenax (modified polyphenylene oxide).

4.3.1. Water extraction

Water extract prepared for toxicity testing

Six of the nominated paper and board samples were intended for wet food contact applications and therefore selected for testing employing water extraction. Each of the seven laboratories conducting toxicity tests within Module I required 125-250 mL water extract from each of the paper/board samples. These water extracts were prepared at one of the Module II laboratories by employing the CEN hot water or cold water extraction procedures, EN 647 and EN 645, respectively, without any modification except for the sterilisation of the water extract obtained.

All water extracts prepared were sterilised in order to avoid any microbial contamination during the transportation to the Module I laboratory and further handling of the extract prior

Table 4. Criteria for the extraction procedures

Criteria type	Criteria in Technical Annex	Criteria obtained	Remarks
Identity of the extractables	The chemical content of the extract should be related to the chemical migration typical for that paper sample in contact with wet, fatty or dry foodstuffs	• Hot and cold water extraction has been used to extract P/B materials intended for contact with moist or aqueous foods. • The solvent 95% ethanol has been used to extract P/B materials intended for contact with foods that contain fat. • Modified polyphenylene oxide ('Tenax') has been used to extract P/B materials intended for contact with dry foods	The chemical content of the migrate/extract is determined by the chemical nature of the simulant/solvent/food placed in contact with the P/B. The solvent/simulants used are tailored to ensure that the chemical content is related to the migrate that can be expected into foodstuffs.
Concentration	The concentration in the extract should be no less than the concentration of migrants in foodstuffs	Chemical analysis of the extracts/migrates obtained has revealed that efficient extraction occurs. In particular, the tests by total immersion are more severe than normal or foreseeable conditions of use in contact with foodstuffs. There is one exception; applications such as tea bag tissue and coffee filter papers for which the extraction conditions are only as severe, but are no less severe, than the possible conditions of use.	A major task has been considerations leading to conclusions and recommendations on the applicability of correction factors – as used in migration tests of plastics – because the tests used here are more severe (elicit higher concentrations in the extract) than normal or foreseeable conditions of use in contact with foodstuffs.
Compatibility	The extract should be homogenous, stable, free from particulates and suitable for subsequent bioassay procedures	• The extracts are mono-phasic mobile liquids that can be mixed easily and will be homogeneous. • Participants have agreed storage conditions (refrigerated or frozen) for the extracts. • The extracts are filtered and so are free of particulates (e.g. cellulosic fibres). • The final solvent vehicle of 95% ethanol has been selected as the most suitable (compatible) for the subsequent bioassay procedures, as judged using the tests for possible non-specific inhibition. For P/B intended for moist/aqueous foods, the vehicle of plain water is suitable.	The microbiological stability of the water extracts was ensured by using ultra-filtration. The chemical stability of the water samples in particular was ensured by storing the extracts deep frozen.

to toxicity testing. The sterilisation of the water extracts was done by filtering the extract using membrane filters. The sterilised extracts were then transferred to sterile glass-bottles. The glass-bottles with the extracts were then immediately sent by post to the Module I laboratories. Sterile filtered water was also supplied to Module I partners as a procedural blank together with the sterile hot water and cold water extracts.

Preparation of cold water extracts

Paper sample (10.0 g) was cut into strips and then extracted by water (200 mL) at room temperature for 24 hours according to the CEN 645 procedure. The water extract was recovered from the paper, transferred to a measuring glass-bottle (250 mL). The paper strips were then washed with cold water (2x10mL) and the washings were combined with the water extract and diluted to the final extract volume 250 mL.

For each paper and board sample several water extraction batches were preformed in parallel and subsequently combined into one batch prior to the sterile filtration step. One aliquot of the sterile filtered water extract was set-aside for determination of the dry matter content (DMC) and for GC-MS analysis of the extracted components. The rest of the sterile extract was divided into several 125 – 250 mL aliquots, transferred to glass-bottles and finally sent to the Module I partners for toxicity testing. A description of the cold-water extracts is given in Table 5.

Preparation of hot water extract

Paper sample (10.0 g) was cut into strips and extracted by hot water (200 mL) at a temperature of 80 ^{o}C for 2 hours in accordance with the CEN 647 procedure. The water extract was recovered from the paper, transferred to a measuring glass-bottle (250 mL). The extracted paper strips were then washed with hot water (2x10mL). The washings were combined with the water extract, allowed to cool down to 23 ^{o}C and then diluted to the final volume 250 mL.

Several water extractions batches were preformed in parallel and then combined into one batch. In order to remove suspended particles this batch was centrifuged prior to the sterile filtration step. One aliquot of the sterile filtered water extract was set-aside for determination of the dry matter content (DMC) and for GC-MS analysis of the extracted components. The rest of the sterile extract was divided into several 125 – 250 mL aliquots, transferred to glass-bottles and finally sent to the Module I partners for toxicity testing. A description of the hot water extract is given in Table 5.

Table 5. Description of the six Nominated paper and board samples and the quantity of extractable matter (dry matter content, DMC) obtained from these samples by employing the CEN cold water and hot water extraction procedures.

Sample No.	**Recycled fiber content**	**Simulant and test conditions**	**Grammage[a]** *(g/m^2)*	**Grammage[b]** *(g/m^2)*	**Extractable matter (DMC)** *(mg/L, water)*	**Extractable matter** *(mg/g, P/B)*	**Extractable matter** *(mg/dm^2, P/B)*
NSP 6	98 %	Water Time:24 h, Temp:20 °C	300	280	244	6.1	17.1
NSP 7	0 %	Water Time:24 h, Temp:20 °C	285	272	48	1.2	3.2
NSP 11	0 %	Water Time:2 h, Temp:80 °C	210	196	472	11.8	23.1
NSP 18	0 %	Water Time:24 h, Temp:20 °C	240	228	312	7.8	18.7
NSP 19	0 %	Water Time:24 h, Temp:20 °C	300	290	132	3.3	9.6
NSP 20	80 %	Water Time:24 h, Temp:20 °C	30	28	112	2.8	0.8

[a] Data from the supplier
[b] Measured at the laboratory

4.3.2. Ethanol extraction

Two procedures were used for ethanol extraction. Total immersion test method was performed on selected samples of the BSP serie and samples NSP4, NSP8 and NSP13 according to the pre-standardization method (ENV 1186-15) employing 95% ethanol as the extraction solvent (food simulant). (The NSP samples were also extracted by the modified procedure described below). Subsequently this method was modified in order to maximize the amount of extractables for the biological tests.

In the ENV 1186-15 procedure, 1 dm^2 paper sample is cut into small stripes (1x3 cm) and placed in a glass cylinder with 50 ml of the extraction solvent and covered with an aluminium foil. The extraction was allowed to proceed without any stirring of the mixture at room temperature (20°C)

for a length of time of 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.

Volume and concentration of ethanol extract needed for toxicity testing

The 7 laboratories conducting toxicity tests within Module I required, collectively, a total of about 80 mL of ethanol extract from each paper/board sample. Because the test organisms used in Module 1 can tolerate only low concentrations of ethanol in the test media (typically up to 2% only) then ethanol extracts prepared from paper/board effectively suffer a large dilution in the tests. Consequently, it was decided to supply a more concentrated ethanol extract to compensate for this subsequent dilution in the aqueous test media. This was achieved by using a high mass/volume ratio of paper/solvent used in the extraction procedure (taking care to avoid any solubility limitations) and also by making a 10-fold concentration of the extract by evaporation (taking care to avoid migrate loss by volatilisation). For each batch of extracts, unused ethanol was concentrated in volume and supplied to Module I partners as a procedural blank.

Preparation of concentrated ethanol extracts

Paper sample (200 g) was cut into strips and extracted by immersion in 95% ethanol (2 L) at room temperature for 24 hours. The solvent was recovered from the paper with minimal mechanical pressing and the extract was filtered through Whatman no.1 filter paper. The approximate volume of solvent recovered was recorded. A portion (100 mL) of each extract was set-aside for the determination of extractable dry matter content (DMC). A second portion of each extract (800 mL) was placed in a one-litre Duran bottle and taken just to dryness under a gentle stream of nitrogen, at room temperature with no external heating. The residue was redissolved in ethanol (80 mL) and portions then sent to the Module I partners for testing. A description of the ethanol extracts is given in Table 6.

Preparation of a low molecular weight fraction of the ethanol extract of NSP4

Paper (188 g) was cut into strips and extracted by total immersion in 95% ethanol (1.95 L) at room temperature for 24 hours. A procedural blank was prepared in the same way (in the absence of the NSP4 paper sample). The ethanol extract was Ultra-filtrated on a membrane of nominal 1000 Da cut-off. A total volume of 500 ml was applied and the permeate low molecular weight fraction

(LMWF) was collected until a volume of 50 mL was obtained. A portion (10 ml) of the LMWF was evaporated to dryness under nitrogen at room temperature. The mass of the residue was recorded and was corrected for the procedural blank sample.

4.3.3. Tenax extraction

Preparation of Tenax

Tenax is conventionally activated by oven heating at ca. 300ºC in air, but it breaks-down to give a constant background bleed in subsequent ethanol extraction (equivalent to ca. 0.5 mg/dm^2). It was considered possible that this background bleed may result in problems with the toxicity tests. Therefore an alternative method of Tenax activation was used. This involved Soxhlet extraction of the Tenax overnight using ethanol, drying under a gentle stream of nitrogen, and then activation overnight under a flow of nitrogen at 150°C.

Considerations of the nature of the extract needed for toxicity testing

Because Tenax is a finely-powdered, insoluble polymer, it is necessary to take the exposed Tenax 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 is a suitable solvent for extraction of Tenax and ethanol is also a suitable vehicle for the toxicity assays. The same considerations for extraction using ethanol (see above) also applied for the samples extracted using Tenax. That is, the total volume of extract needed to satisfy the Module 1 tests was 80 mL and the extract needed to be pre-concentrated to compensate for the dilution effect brought about by adding only a small proportion to the *in vitro* assays. It was calculated that a preparation equivalent to 0.2 dm^2/mL would place the extracts at the lower end of the ethanol extracts of NSP4, 5, 8 and 13, which were prepared in the range 0.15 to 0.71 dm^2/ml (see Table 6).

Table 6. Description of the concentrated ethanol extracts supplied to Module I partners

Sample No.	Sample Description	Grammage (g/dm^2)	Paper equivalents g/mL ethanol	Paper equivalents dm^2/mL ethanol
NSP4	Board GD3	3.00	1.00	0.333
NSP5	Board GD	5.00	1.00	0.200
NSP8	White top kraftliner	1.40	1.00	0.714
NSP13	Corrugated board (virgin)	6.66	1.00	0.150

Preparation of the Tenax extracts in a final vehicle of ethanol

Twelve circles of paper/board (each 138 mm in diameter, giving total exposed area of 18 dm^2) were stacked in a glass Petri dish along with Tenax (6 Tenax beds of 3 g each) making single-sided contact with the paper/board. The schematic in Figure 3 depicts the arrangement, with the 6 beds of powdered Tenax shown in green. The lid was fitted and the dish was stored in an oven to give the required time and temperature exposure conditions (see Table 3).

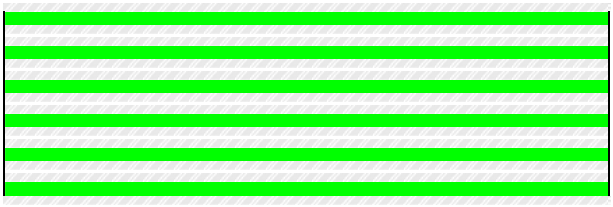


Figure 3. Schematic for the tests using Tenax

After the exposure period the sample circles were removed using tweezers allowing the exposed Tenax powder to fall into the dish with minimal brushing-off if required. The exposed Tenax was then extracted with ethanol (3 x 100 ml) and the combined extracts were concentrated to a volume of 80 ml under a gentle stream of nitrogen. This provided an extract at 0.22 dm^2/mL. For each batch of Tenax tests, unexposed Tenax was similarly extracted with ethanol, concentrated in volume, and supplied to Module I partners as a procedural blank. A description of the concentrated ethanol extracts of the Tenax sent to the Module I partners is given in Table 7.

Table 7. Description of the concentrated extracts of the Tenax, supplied to Module I partners

Sample No.	Sample Description (from sponsor)	Grammage g/dm^2	Paper equivalents g/mL ethanol	Paper equivalents dm^2/mL ethanol
NSP1	Solid board	6.65	1.50	0.225
NSP2	Board GD2	3.00	0.675	0.225
NSP3	Board GD2 high newsprint	3.00	0.675	0.225
NSP9	Unbleached liner recycled	1.70	0.383	0.225
NSP10	Bleached liner recycled	1.40	0.315	0.225
NSP12	Recycled fluting	1.05	0.236	0.225
NSP14	Corrugated board	5.00	1.13	0.225
NSP15	Kraft paper virgin	0.70	0.158	0.225
NSP16	Paper based on de-inked fibre	0.40	0.0900	0.225
NSP17	Folding boxboard	2.55	0.574	0.225

4.3.4. Spiked extracts

Acrylamide spiking experiments

A cold water extract from recycled test liner (BSP5), prepared according to EN 647 standard, and an ethanol extract from bleached kraft (BSP9), prepared according to pre-standardization method ENV 1186-15, were used in spiking experiments with acrylamide. The total content of migrants in the respective final extracts was 16.1 g l^{-1} (BSP5) and 46 g l^{-1} (BSP9). These extracts were selected because of their low response in preliminary cytotoxicity assays.

Acrylamide was added to the extracts at various concentrations covering the toxicity range of the chemical dissolved either in water or ethanol. The cytotoxicity assays and comet assays were then performed according to the standard procedures using both water, ethanol and the paper and board extracts as controls and water or ethanol solutions of acrylamide as positive controls.

Cereulide-spiked paper and board samples

Experimental handsheets were made in a laboratory paper making equipment (dynamic drainage analyzer (DDA), described by Forsberg and Bengtsson, 1990, Fig. 1). The pulp slurry contained 340 g of kraft pulp slurry (10.6 g L of kraft $pulp^{-1}$, SR 20) and 260 g of water (containing 200 mg of Na_2SO_4 L^{-1}) making a total of 600 ml of slurry needed for making handsheets with the DDA. Five ml or 0 ml of *B. cereus* suspension (12 g of plate grown bacterial biomass L sterile $water^{-1}$, viable or autoclaved) was added into the pulp slurry (location A in Fig 1) and stirred for 30 sec at 1000 rpm. The handsheets (3.8 g / 0.8 dm^2) were dried using a photographic dryer (JET-2 Dryer, Japo Co Ltd, Japan) at 80 °C to dry weight of 5 g dm^{-2} and stored wrapped in aluminum foil in the dark.

The 4 d plate grown biomass of *B. cereus* strain NS58 contained 2 μg of cereulide mg^{-1} wet weight. The biomass (6 g) was suspended in 510 ml of water and autoclaved (15 min in 121 °C) to inactivate viable cells. The pulp slurry (stock, 600 ml) was spiked with 5 ml of the autoclaved *B. cereus* NS58 suspension, containing 60 ± 5 μg of cereulide. This dose was equivalent to 10^6-10^7 *B. cereus* cells (spores and vegetative cells) per ml of stock.

For assessing leaching of cereulide from paper, 1 up to 3 spiked handsheets were extracted into hot water (CEN EN 647:1993), cold water (CEN EN 645:1993) or leached into ethanol (CEN PrEN

1186-15:2001) as described in the respective standard / pre-standard protocols and in Isberg *et al*. (2002). The extracts obtained were used for cytotoxicity testing.

Benzo(a)pyrene B[a]P spiked samples

As recommended by the ad-hoc expert group during the meeting held in Barcelona (17 november 2004), paper extracts spiked with low amounts of mutagens were tested. Two extracts NSP 11 (a water extract) and NSP 4 (ethanol extract) were selected. These extracts were spiked with B[a]P at different concentrations and with or without DIPNs at 80 µg/ml DMSO, which is the limit of solubility of this compound in the culture medium. DIPNs was used to detect any eventual interaction of DIPNs in the xenobiotic metabolising pathways involved in the activation of B[a]P.

4.4. Analytical procedures and fractionation

4.4.1. Chemical analysis of the water extracts

Determination of the dry matter content (DMC) of the extracts

The determination of the dry matter content of the extracts was done according to the CEN 920 procedure. A portion (100 mL) of the water extract was poured into a pre-weighed aluminum dish, placed on a heating plate and evaporated until the residue was almost dry. The residue was then brought to final dryness by placing the aluminum dish with the extract residue in a ventilated cabinet at 105 C for 30 min. The dish with the residue was then put into a desicator and cooled down to room temperature. Finally, the mass of the extract residue (dry-matter content of the extract) was determined gravimetrically.

Gas chromatographic and mass spectrometric (GC-MS) analysis

For the GC/MS characterization an aliquot (10 mL) of the water extract was placed in a 20 ml glass vial equipped with a Teflon cap. A mixture of two internal standards (14-methylpentadecanoic acid and cholestanol) was added for the purpose of quantification. The water was then removed by evaporation to near dryness of the extract residue by employing a gentle stream of nitrogen gas. The dry extract residue was then immediately re-dissolved in 1 mL dry acetone (pro analysis) thereby making the sample ready for the derivatization step.

The derivatization of the extract resides dissolved in dry acetone (1.0 mL) was performed by treatment with the silylation reagent BSTFA (0.70 ml) [N,O-bis(trimethylsilyl)trifluoro-acetamide]. The Teflon cap was screwed on and the reaction mixture was kept at 70 ºC for 30 minutes. The mixture was then taken to room temperature. The solvent and excess of the reagent were subsequently removed by evaporation under a nitrogen gas-stream. Finally, the derivatized extract residue was dissolved in 1ml of dichloromethane (pro analysis) and subsequently analysed by GC/MS.

The silylated extracts were analysed by employing a GC-MS instrument equipped with a VF-5ms phase fused silica column or a BPX5 bonded phase fused silica column. The mass spectrometer (HP5989 MS-Engine) was operated in full scan mode with one scan, 50 to 600 m/z, per second.

Preparative GPC fractionation of BSP6 and BSP7 cold-water extracts

The cold-water extract of BSP7 and BSP6 already prepared and analyzed, were fractionated in sub-fractions a) and b) corresponding respectively to High (collected from 10 to 21 minutes) and Low MW (collected from 21 minutes to 37 minutes) fractions. All injections were performed in duplicate in order to collect a significant volume of sub-fractions. A total of 16 sub-fractions were collected.

4.4.2. Chemical analysis of the ethanol extracts

Determination of the dry matter content of the extracts

A portion (100 mL) of each raw (unconcentrated) extract was evaporated to dryness under nitrogen in a pre-weighed vial, at room temperature with no external heating. The mass of the residue (dry-matter content of the extract) was recorded and corrected for the analytical blank which was 4.6 mg/L.

GC-MS analysis

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

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 a nominal concentration of 20 µg/ml in the extract and the resulting solution was analysed by GC-MS. The instrument used was an Agilent MSD 5973inert, fitted with a DB-5ms column (5% diphenyl – 95% dimethyl polysiloxane, 30 m x 0.25 mm i.d, 25 µm film thickness) and operated with electron impact ionisation and in full scan mode (m/z 40 - 450). The column was held at 40°C for 3 minutes, raised to 280°C at 10°C/minute, where it was held for 5 minutes. The injection (1 µl) was splitless (1 minute) at 250°C, with a transfer line temperature of 280°C.

Derivatisation and GC-MS analysis

A portion (1 ml) of extract was pipetted into a 20 ml glass vial. Internal standard was added (20 µl of a 2 mg/ml solution of hexadecanoic acid). The vial was placed under nitrogen flow to evaporate to near dryness. The residue was dissolved in 1ml of dry acetone and 0.7 ml of BSTFA (with TMS) was added and the vial capped. After reaction in a 70°C oven for 30 minutes to derivatise, the vial was allowed to cool, the cap removed, and the vial placed under nitrogen flow to evaporate to near dryness. 1 ml of dichloromethane was added to dissolve the residue. The solution was then analysed by GC-MS.

4.4.3. Chemical analysis of the Tenax extract

The Tenax extracts in a concentrated ethanol vehicle were analysed by GC-MS both with and without derivatisation, as described above.

4.5. Toxicological testing

4.5.1. Cytotoxicity tests

A. Cytotoxicity assays with cultured mammalian cells

RNA-synthesis inhibition test

The i*n 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 minutes 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. With both the cell types reduction more than 70% was considered clearly positive response.

a) The automated test procedure using HepG2 cells

This protocol was already described by Valentin *et al.* (2002) 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 x 10^4 cells in 0.2 ml of the 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) was added to each well containing 50 µl of cells and medium. Uridine incorporation was stopped by adding 3% (w/v) SDS (sodium dodecyl sulfate) (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 had been wetted with 100 µl of 20% TCA just before use. After the application of samples, TCA (100 µl/well) was added again to individual wells to maximise precipitation of nucleic acids and proteins. After 5 minutes of contact between TCA and cell lysate, the microplate was vacuum filtered using a Manifold system (Millipore) and washed with 200 µl/well of ethanol. After 2 hours drying in an oven (< 60°C), the plate was counted directly in a Top Count microplate reader (Packard, Rungis, France).

b) The 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 % NEAA (Euroclone, Weatherby, UK) and 5 % FBS. The cell density at the beginning of a 17 h

exposure to the test agent was 6 x 10^5 ml^{-1}. The 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 uptake times, 50 µl subsamples were removed into a 96–well microplate and mixed with 30 µl of 3 % SDS previously pipetted into the wells. The TCA-precipitation, filtering, washing with ethanol and drying was done as described for the HepG2 cells. The radioactivity was measured using a Wallac Multiscreen Cassette and a MicroBeta-top count microplate reader (WallacPerkin Elmer Turku, Finland) with 25 µl of the scintillation liquid (Optiphase SuperMix Cocktail, Wallac Perkin Elmer, Turku, Finland) per well.

Cytotoxicity tests with human larynx carcinoma cell line (HEp-2)

For the tests the cells were routinely cultivated as a monolayer at 37 °C and in 5 % CO_2 atmosphere in MEM (Euroclone Ltd, Weatherby, UK) medium supplemented with Eagle salts, 5 % FBS (Sigma, St. Louis, 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 $9x10^3$ 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 $x10^5$ cells/dish and, when semiconfluent, treated with different concentrations of the test substance. The following parameters were used to evaluate the toxicological endpoints. More than 20% reduction in each of these parameters compared to controls was considered as indication of toxicity.

a) 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 PBS. The cells were then incubated with the neutral red dye (50 µg/ml medium) at 37 °C for three hours. After that time, neutral red solution was removed and the wash/fix solution (0.5% formaldehyde, 1% $CaCl_2$) was added into each well, gently shaking the plate for two minutes. 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 minutes at room temperature. The measurement of absorbance at 540 nm was performed by a microplate reader (Microplate Reader, Model 450, Bio-Rad Laboratories, Milano, Italy).

b) Total Protein Content (TPC)

The same plates used for NRU assay were then washed twice with PBS and the TPC assay was performed after solubilisation 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: 1mg/ml in 0.5 N NaOH).

c) 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 one 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 10 cells were counted (Wilson, 1992) by a Colony Counter (Stuart Scientific, U.K.).

Cytotoxicity tests with the mouse haepatoma 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, St.Louis, USA) 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 x 10^4 cells/ml). 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 24h exposure the cells were washed twice with PBS-buffer. Subsequently 50 µl of sodium phosphate buffer (0.05 mM, pH 8.0) was added to each well before freezing the plates for at least 15 min (-70°). The plates were thawed for 15 min before a further addition of 150 µl of sodium phosphate-buffer into the wells followed by 50 µl of 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 one minute. 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. More than 20% reduction of total protein content was considered toxic.

B. Cytotoxicity with the boar spermatozoan motility inhibition test

The test was performed as described by Andersson et al. 1998. 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 to 4 days at room temperature. Each day the contents of the tubes were mixed by overturning once. The sperm motility was monitored daily and compared to 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 (a) adding 20 µl of each concentration of the test substance to 2 ml of the semen and subsequently diluting each toxic concentration two-fold, until a non-toxic dilution was obtained, or by (b) diluting the test substance in the extended boar semen according to the instructions indicated.

C. The bioluminescence test as cytotoxicity assay

The test was performed by the method of Jokinen et al. (2001), modified from the ISO standard 11348-1:1998(E). The modifications involved exposure temperature (25°C instead of 15°C used in ISO standard) and adapting the protocol for an automated instrument (BioOrbit 1251 Luminometry System, Turku, Finland). *Photobacterium* (*Vibrio) fischeri* (DSM 7151) was obtained from Deutsche Sammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany). *P. fischeri* suspensions for the test were taken from agar plates, made by adding agar in The Liquid Growth Medium and pretested for luminescence as prescribed in the ISO11348. The bacterial extracts, diluted in ethanol in steps of 1:1 to reach the wanted end dilution, were used to expose the *P. fischeri* suspension, prepared from 2 to 5 d grown plate culture in the Test medium of ISO11348, containing 4 mM of K^+ and 341 mM of Na^+ in the final exposure mixture. Ethanol was used as negative control, two at the beginning of the test and one at the end of the run. Cr^{VI} was used as the standard reference as recommended by ISO 11348. Light emission of the cuvettes measured after 5, 15 and 30 min. The EC_{50} values were computed with Excel software.

4.5.2 Genotoxicity tests

Ames test

Ames test is a mutagenicity test based on histidine auxtrophic strains of *Salmonella typhimurium* (Maron and Ames, 1983). These strains need histidine in their growth medium in order to grow and form typical bacterial colonies on culture plates. If, however, a reverse mutation occurs restoring

the ability to synthesize histidine, the mutant bacterium starts dividing forming a revertant colony. Thus, the number of colonies formed by bacteria exposed to the test chemical reflects the mutagenic activity of the test agent. In practise, the bacteria and test agent are mixed in a small amount of soft agar, and the mixture is poured on a plate containing histidine sufficient only for a few cell divisions. Regularly also a liver microsomal fraction (S-9 preparation) obtained from a rat exposed to a chemical inducing the drug metabolizing enzymes is included in order to metabolically activate the test compound. After a 48-72 h incubation the colonies are counted, and the mutagenic activity estimated. If the number of colonies in plates containing the test agent is at least doubled in comparison to spontaneous revertant numbers, the test chemical is considered mutagenic.

The routinely used strains include *S. typhimurium* TA 1535 and TA100, which detect base change mutations, and the frame-shift mutant strains TA 1537 and TA98. TA100 and TA98 both contain a plasmid PKM101 which greatly sensitise them to mutations. Strains TA97 (TA1537 + pKM101) and TA102 (histidine mutation in a multicopy plasmid + pKM101), the latter of which is specially sensitive to oxidative mutagens, have been included in the test battery. The metabolic activation system applied was a commercial preparation (IFFA CREDO, L' Arbresle, France). Additionally S-9 preparation obtained from phenobarbital-β-naphtoflavone-induced rat liver was prepared by the National Laboratory Animal Center, University of Kuopio.

Comet assay

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). The test is used to screen agents with genetic activity, i.e., possible environmental mutagens/carcinogens. Primary cells or cells of an established cell line in an exponential stage of growth are exposed to the test substance with and without metabolic activation. When metabolically competent cells are used (e.g. HepG2 cells), no additional metabolic activation system is needed.

For the test, the cells are 1) treated with the test chemical, 2) mixed in low-melting point agarose, 3) spread on slides precoated with normal-melting point agarose, 4) lysed, 5) run in a horizontal electrophoresis tank, 6) stained, and 7) analysed for the comet parameters, i.e., for the migration of DNA after the electrophoresis. Commercial image analysis systems are available for the analysis.

HepG2 cells are grown for the SCG assay at 37 °C in a 5% CO_2 atmosphere. The culture medium is EMEM supplemented with 2 mM L-glutamine, 1% non-essential L-amino acids (NEAA), and 10%

heat-inactivated foetal bovine serum (FBS). For the test, wells of a 96-well tissue culture plate are seeded with 5 x 10^4 cells in 0.2 ml of the culture medium. After 28 and 44 hrs of incubation, the cells are treated with the test substance, and the negative and the positive control chemicals. The cells are exposed for 20 hrs and 4 hrs in EMEM supplemented with 0.5% FBS.

At least three adequately spaced concentrations of the test substance are used. The highest concentration should not induce excess cytotoxicity (decrease viability, compared to the control cultures, by more than 30%).

After the exposure, the cells are washed and harvested with trypsin-EDTA. Six wells (of a 96-well tissue culture plate) treated with the same concentration are pooled, centrifuged, and resuspended in phosphate buffered saline solution (PBS). Twenty µl of cells (10 000 cells) are mixed in 75 µl of low-melting point agarose (LMP), spread on slides precoated with normal- melting point agarose (NMP) and lysed in ice-cold lysing solution. An electrophoresis is done, followed by staining of DNA and analysed.

Olive tail moment (a measure of tail length x a measure of DNA in the tail) is used as the metric to characterize the DNA damage in individual cells. The analysis is done using an automated image analysis system (Komet, version 4; Kinetic Imaging, UK). A total of 50 individual comets are analyzed

Micronucleus test

The *in vitro* micronucleus (MN) assay is a short-term test to study the induction of damage to the chromosomes in cultured cells (Miller et al. 1998). The test is used to screen agents capable of inducing structural or numerical changes in the chromosomes. Micronuclei are small, extra nuclei seen in interphase cells. Micronuclei are formed during the metaphase/anaphase transition of mitosis (cell division) from 1) acentric chromosome fragments as a result of chromosome breakage, or 2) they may arise from whole chromosomes as a result of malfunction of the mitotic apparatus.

Primary cells or cells of an established cell line in an exponential stage of growth are exposed to the test substance with and without metabolic activation. When metabolically competent cells are used (e.g. HepG2 cells), no additional metabolic activation system is needed. For the test, the cells are treated with the test chemical and further cultured in the presence of cytochalasin-B (Cyt-B). Cyt-B prevents cytokinesis, and if micronuclei are formed they remain in the same cell with the daughter nuclei. Accordingly, the analysis of micronuclei is done, with the cytokinesis-block technique, in

binucleated cells (second cycle interphase cells) that have divided once in culture after the induction of the original damage.

HepG2 cells are grown at 37 °C in a 5% CO_2 atmosphere. The culture medium is EMEM supplemented with 2 mM L-glutamine, 1% non-essential L-amino acids (NEAA), and 10% heat-inactivated foetal bovine serum (FBS). For the test, wells of a 6-well tissue culture plate are seeded with 1.5 x 10^6 cells in 3 ml of the culture medium. After 24 hrs of incubation, the cells are treated with the test substance, and the negative and the positive control chemicals. The cells are exposed for 20 hrs or 4 hrs in EMEM supplemented with 0.5% FBS. At least three adequately spaced concentrations of the test substance are used. The test concentration should cover a range from maximum to little or no cytotoxicity. After the treatment, the cells are washed and further incubated in complete culture medium supplemented with cytochalasin B (final concentration 4.5 μg/ml) for 30 hrs.

For the harvest, the cells are washed in PBS and allowed to recover in complete culture medium for 1.5 hrs. Before sampling, the morphology and the growth of the cells is checked. After this, the cells are swelled by incubation in EMEM diluted 1/1 (v/v) with PBS for 5 minutes. The cells are then harvested with trypsin-EDTA and fixed for 15 min with acetic acid/ethanol (1/3) (v/v) diluted in EMEM with 10% FBS. A second fixation is performed with acetic acid/ethanol (1/3) (v/v).

The fixed cells are spotted on glass slides and stained with May-Grünwald diluted 1:1 (v/v) in Sorensen buffer pH 7 for 8 min, followed by Giemsa (diluted 1:10 in the same buffer (v/v)) for 25 min. The slides were then rinsed with tap water and air-dried. Micronuclei are scored in binucleated cells (BNC) using a light microscope. The following criteria are used in the analysis: 1) the diameter of a micronucleus has to be less than 1/3 of the diameter of the main nucleus; 2) the micronucleus has to be surrounded by a clear membrane 3) without touching the main nucleus.

A compound is considered positive if at least a two fold increase in the number of micronucleated cells is observed at one or more concentrations. Statistical analysis (non-parametric Mann-Whitney U-test) is used as an aid in the analysis of the results per slide and two slides are analyzed per concentration.

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5. RESULTS

5.1. Performance of bioassays on model compounds and contaminants

5.1.1. Cytotoxicity assays

Compatibility of the test systems with organic solvents

As a preliminary to the testing program, toxicity tests were performed on different solvents that could be considered as final solvents for extracts. The results are given in Table 8.

Table 8. The maximum solvent concentrations compatible with the various short term cytotoxicity tests of the project

Solvent	Non-toxic concentration in different tests (%, v/v)					
	Acute cytotoxicity tests		Sublethal cytotoxicity tests			
	Mouse hepatoma cell line (Hepa-1)	Human larynx carcinoma cell line (Hep-2)	Boar spermatozoan motility inhibition test	RNA-synthesis inhibition test		Bioluminescence test (EC_{50})
				HepG2 cells	Hela cells	
Ethanol	2	1	2	2	0.5	15
Methanol	1	1	2	> 2	> 0.5	12.5
DMSO	0.5	1	1	2	0.5	> 10
Acetone	> 2	> 8	1	>2	>0.5	7.5
Hexane	>2	-	1	> 2	-	0.06

(On the basis of these results, ethanol was chosen as the solvent for paper and board samples intended for fatty foods and also as the final solvent in Tenax extractions)

Regarding the genotoxicity assays, the maximum concentrations tolerated by HepG2 cells could also be applied in the Comet assay, which was performed using this same cell line. In Ames tests the maximum amounts were 200 µl per plate (only ethanol, methanol and DMSO).

Cytoxicity tests on model compouds

The relative toxicities (EC_{50}) of the used substances (potassium dichromate, DMSO and 2',5' dimethoxy-acetophenone) in the test systems applied are summarized in Table 8. **2',5' dimethoxyacetophenone** 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 *P. fischeri* in a roughly dose-dependent fashion. The EC_{50} values for *P. fischeri*, calculated from different test samples ranged between 0.05 and 0.12 μM. In the case of **potassium dichromate**, the most sensitive tests system was the *P. 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 end point, 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 9). In the *P. fischeri* assay the EC_{50} values ranged between 0.0058 – 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 M 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 test and *P. fischeri* test, only the top dose gave a clearly toxic response.

Table 9. The relative toxicities of the test substances in the test systems applied.

Compound	EC_{50} – values					The lowest tested concentration giving a toxic response
	Cytotoxicity assays		RNA-synthesis inhibition assay		Bioluminescence test	
	Hep-2 cells	Hepa-1 cells	Hela cells	HepG2 cells	*P. fisheri*[2]	Boar spermatozoan motility inhibition assay
Potassium dichromate [µM]	1.9 (NRU) 5.8 (CFA)	< 3.9[1]	5.4	8.0	0.0067	Non-toxic at every tested concentration
DMSO [M]	0.55 (NRU) 0.53 (CFA)	0.29	0.19	0.28	0.02	0.7
2',5'-dimethoxyacetophenone [mM]	3.1 (NRU)	0.69	0.28	1.33	0.00008[3]	0.8
Cereulide [nM] [4]	1.0 (NRU) 2.0 (CFA)	0.8	-	2	170	0.2-0.4

[1] The extrapolation of EC_{50} not possible, because of the total cell death at the lowest tested concentration
[2] Mean of the EC_{50} – values obtained from different tests concentrations
[3] Calculated from the test sample with the highest DMSO concentration
[4] The extract of NS61 (a cereulide non-producer) was consistently non-toxic in all the assays (data not shown); it was necessary to use purified cereulide instead of crude extract to obtain results in the bioluminescence test.

The performance of the RNA-synthesis inhibition assay in different laboratories

During the project one of the aims was to introduce the RNA-synthesis inhibition test (performed on the HepG2 cell line) to UKU and ISS. The test was originally developed in ENSBANA. To ensure the optimal performance of the test in different laboratories and that the eventual results are comparable, a ring test exercise between the three laboratories was arranged using model chemicals (potassium dichromate, 2',5'-dimethoxyacetophenone and DMSO, prepared by ENSBANA).

The results are summarised in Table 10. It can be seen, that there was a reasonable agreement between the three laboratories in the EC_{50}-values for the model chemicals. However, for unknown reasons, the recovered counts from the radiolabelled cells was consistently lower in ISS than in ENSBANA or UKU. Consequently, it was decided that this test will be used in ENSBANA and UKU only, during the project.

Table 10. The EC_{50}-values of model compound in the RNA-synthesis inhibition assay with HepG2 cells, performed in different laboratories.

Laboratory	IC_{50}		
	Potassium-dichromate [µM]	2',5'-dimethoxy-acetophenone [mM]	Dimethyl-sulphoxide [M]
ENSBANA	6,4	1,3	0,18
UKU	7,6	0,9	0,25
ISS	7,5	1,7	0,22

5.1.2. Genotoxicity assays

Comet assay

Acrylamide, 2,4-diaminotoluene and benzo(a)pyrene were positive in the comet test as well as nitroquinolineoxide and methylmethanolsulfonate which were used as positive controls. The results were in good qualitative agreement between laboratories ENSBANA and NPHI. Representative results are given in Figure 4.

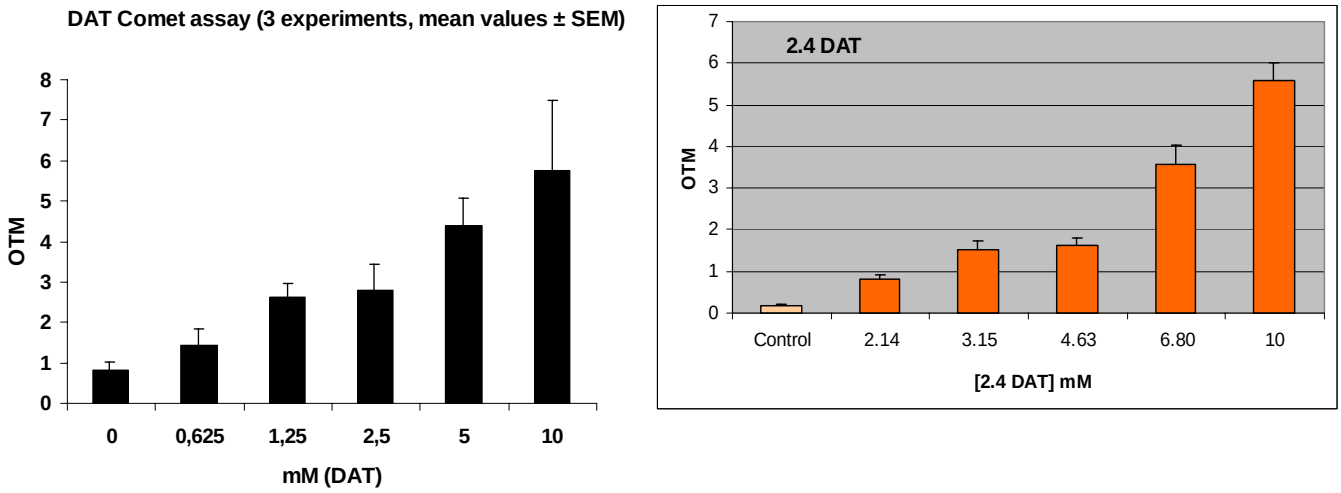

Figure 4. 2,4 Diaminotoluene in comet assay. Results from NPHI (left panel) and ENSBANA (right panel). The figures show a good qualitative agreement between the two laboratories.

Micronucleus test

Acrylamide, 2,4-diaminotoluene, benzo(a)pyrene, nitroquinolineoxide, methylmethanolsulfonate were all positive performed by ENSBANA. (Figure 5). However the results could not be reproduced in NPHI (data not shown).

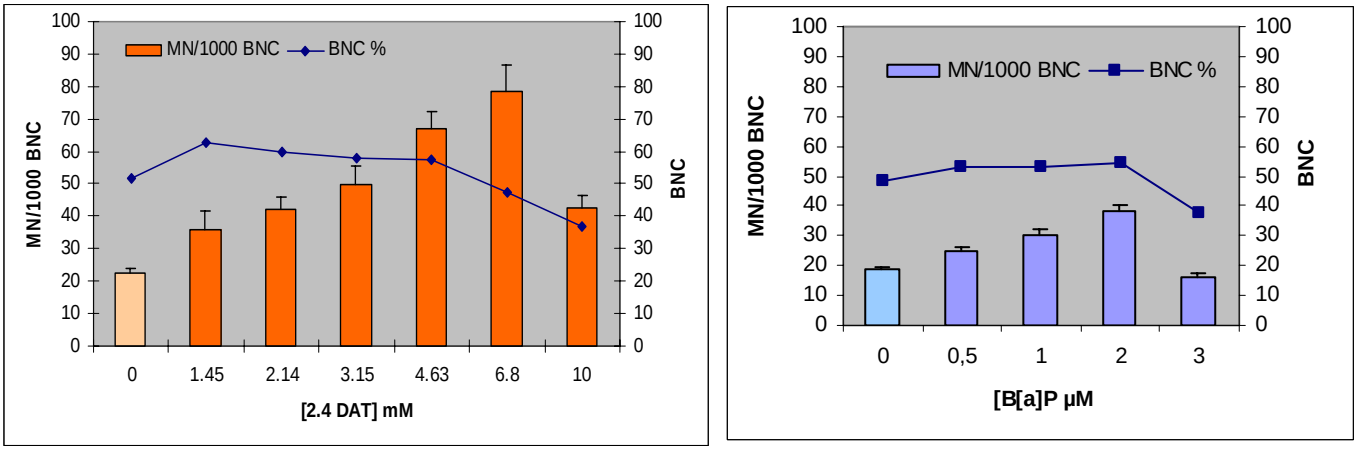

Figure 5. Representative results of the micronucleus tests performed on 2,4-diaminotoluene (left panel) and benzo(a)pyrene (right panel).

5.1.3. The provisional breakpoints of the different assays

In order to facilitate the interpretation of the different tests, the following provisional breakpoints for positive and negative responses in cytotoxicity assays were agreed, as well as colour codes to be used in tabular presentations (Table 11). In the subsequent tables, the use of different colours refer to this convention, unless otherwise indicated. **With genotoxicity assays the only colour codes are red (positive) and green (negative).**

Table 11. The provisional breakpoints of the different assays, and the corresponding colour codes

Test	Positive	Marginally positive	Non-significant effect	Negative
Acute cytotoxicity	50% reduction of viability (EC_{50}) obtained	Reduction of viability > 20% but < 50%	Up to 20% reduction of viability (EC_{20})	< 20 % reduction of viability
RNA-synthesis inhibition	> 70% reduction of RNA-synthesis	> 40% but < 70% reduction of RNA synthesis (EC_{50} obtained)	> 40 % reduction of RNA-synthesis at the top dose (no EC_{50} obtained)	< 30% reduction of RNA-synthesis
Boar spermatozoan motility inhibition	Sperm motility still inhibited at dilution 1/ 4 of the sample	Sperm motility still inhibited at dilution 1/2 of the sample	Inhibition by the top dose (undiluted sample) only	No inhibition of motility at any tested concentration

5.2. Tests on wood related compounds and chemical contaminants

The results obtained with wood-related substances and model contaminants in cytotoxicity and genotoxicity assays are given in Table 10. In the table the concentration ranges used in the cytotoxicity test and comet assay are indicated. It should, however, be noted that in the boar spermatozoan motility inhibition assay the maximum concentrations were limited by the total volume of sample allowed in the system (2%). Also in the Ames test the concentrations were not related to those used in cytotoxicity tests (or comet assay) but were those that were tolerated by the tester strains when applied in plates and are listed below.

Ames tests were performed on the following project chemicals:

- Acrylamide (10 µg – 10 mg/plate)
- 2,4-Diaminotoluene (2,5 - 2000 µg/plate)
- Phthalate mixture (5 µg – 500 µg/plate)
- DiPNs (5 µg – 500 µg/plate)
- Blancophor disulphone (5 µg – 500 µg/plate)
- Blancophor tetrasulphone (5 µg – 500 µg/plate)

Nitroquinoline 4 –oxide (1 µg/plate) and Benzo(a) pyrene (5 µg /plate) were used as positive controls as well as sodium azide (5µg/plate). The tester strains were TA97, TA98, and TA100.

Table 12. Cytotoxic and genotoxic properties of wood-related substances and model contaminants.

Chemical or extract	Concentration range	Cytotoxicity					Genotoxicity	
		Photobacter fischeri test	Boar spermato-zoan motility inhibition	RNA-synthesis inhibition (HepG2 cells)	Acute cytotoxicity		Comet assay*	Ames test **
					Hepa-1 cells	HEp-2 cells		
Dehydroabietic acid	0.25 - 2 mg/ml	2.6 µg					neg 12,5 µg	neg 800µg
Spruce wood (ethanol extract)	0.25 - 2 %	EC50 ≈ 0.6		EC50 ≈ 1.3 EC40≈ 1.0				nt
Spruce wood (water extract)	1 - 90 %	EC50 ≈ 7.9		EC50 ≈ 33 EC40≈ 25				nt
Acrylamide	1 - 16 mM		5	EC50 ≈ 4 EC40≈ 3.2	EC50 ≈ 2.5 EC20≈ 2	EC50 ≈ 4 EC20 ≈ 1	1.5	neg 10mg
2,4-Diamino-toluene	1 - 45 mM		1	EC50 ≈ 4.3 EC40≈ 2.4	EC50 ≈ 1 EC20 ≈ 0.1	EC50 ≈ 22 EC20 ≈ 5	1	5µg
Phthalate mixture	7 - 150 µg/ml	EC50 ≈ 33	8	EC50 ≈ 99 EC40 ≈ 83	EC50 ≈ 13 EC20 ≈ 9	EC50 ≈ 2.5 EC20 ≈ 2	neg. 800µg	neg 500 µg
Diisopropyl-naphthalene (DiPNs)	2.5 - 74 µg/ml		2.5	EC50 ≈20 EC40 ≈35	EC50 ≈15 EC20 ≈ 10	EC50 ≈ 13 EC20 ≈ 1.4	neg 80µg	neg 500 µg
Blankophor disulphone	0.1 - 12 mg/ml		0.2	EC50 ≈ 0.7 EC40≈ 0.5	EC50 ≈ 1.4 EC20 ≈ 0.9	EC50 ≈5.0 EC20 ≈0.1		neg 500 µg
Blankophor tetrasulphone	1 - 46 mg/ml		0.4	EC50 ≈ 5.6 EC40 ≈ 3.7	EC50 ≈ 5.5 EC20 ≈ 2	EC50 ≈ 19 EC20 ≈ 1		neg 500 µg

*The concentration on the box is the highest tested if different from the top concentration given in the second column.

** The concentrations used in Ames tests were the highest amount per plate not causing the growth inhibition of the tester strains (see text)
nt = not tested

= Positive response (EC_{50} obtained within the concentration range, or spermatozoan motility inhibition at severalfold diluted samples)

= Marginal positive effect (no EC_{50} obtained within the concentration range, spermatozoan motility inhibition at the top tested dose only)

= Negative response

5.3. Results from tests on paper and board extracts

5.3.1. Analytical data on paper and board samples

5.3.1.1. BSP-series

The dry matter contents of the aqueous extracts of the BSP samples are summarized in Table 13. and those of ethanol (and iso-octane) extractions in Table 14.

Table 13. Determination of dry matter content in aqueous extracts prepared according to the cold water and hot water extraction procedures.

Sample code	Type	Grammage (g/m^2)	Cold water extract[a]		Hot water extract[c]	
			(mg/g)[b]	(mg/dm^2)[b]	(mg/g)	(mg/dm^2)
BSP1	Filter paper	13.3	N.a.	N.a.	8.2	1.0
BSP2	MG-Paper	71,5	1.3	0.9	N.a.	N.a.
BSP3	Greaseproof paper	41	19.0	7.8	N.a.	N.a.
BSP4	Corrugated board	727	22.2	153	N.a.	N.a.
BSP5	Test liner	240	6.9	15.5	N.a.	N.a.
BSP6	Fluting	170	41.9	71.0	N.a.	N.a.
BSP7	Chipboard	406	7.0	26.8	N.a.	N.a.
BSP8	White lined chipboard	497	5.6	26.6	N.a.	N.a.
BSP9	SBS	199	N.a	55.6	N.a	N.a

N.a.= Not applicable, a) EN 645, cold water extraction, room temperature for 24 hr, b) mg of extractables per g or dm^2 sample, c) EN 647, hot water extraction, at a temperature of 80ºC and for 2 hr

Table 14. Contents of extractable organic matter analyzed by the total immersion procedure employing isooctane or 95% ethanol as the food simulant.

Sample code	Type	Grammage (g/m^2)	Isooctane extract[a]		95% Ethanol extract[c]	
			(mg/g) [b]	(mg/dm^2)[b]	(mg/g)	(mg/dm^2)
BSP1	Filter paper	13.3	N.d.	N.d.	N.d.	N.d.
BSP2	MG-Paper	71,5	1.2	0.9	3.4	2.4
BSP3	Greaseproof paper	41	N.d.	N.d.	1.8	0.8
BSP4	Corrugated board	727	1.7	11.3	5.7	38.7
BSP5	Test liner	240	2.0	4.7	4.8	11.7
BSP6	Fluting	170	0.3	0.6	5.2	9.3
BSP7	Chipboard	406	3.7	15.2	4.9	19.9
BSP8	White lined chipboard	497	4.0	20.0	6.3	31.4
BSP9	SBS	199	0.9	N.a	2.3	N.a

N.d.= Not detectable, <0.5 mg/dm^2 , N.a = Not applicable, a) Total immersion test, isooctane at room temperature for 24 hr, b) mg of extractables per g or dm^2 sample, c) Total immersion test, 95% ethanol at room temperature for 24 hr

Figure 6. depicts the constructed gas chromatogram obtained by py-GC-MS analysis of the hot-water extract from the sample BSP 1. This chromatogram shows that the extract was composed predominately of (water-soluble) carbohydrates; with only small amounts of lignin derived oligomeric compounds being detected. In contrast, the corresponding analysis of the cold-water extract from sample BSP6 demonstrated mainly peaks originating from lignin-derived compounds. Hence, the compositions of these water-extracts were dissimilar, but in both cases dominated by oligo- and polymers derived from the wood fibers.

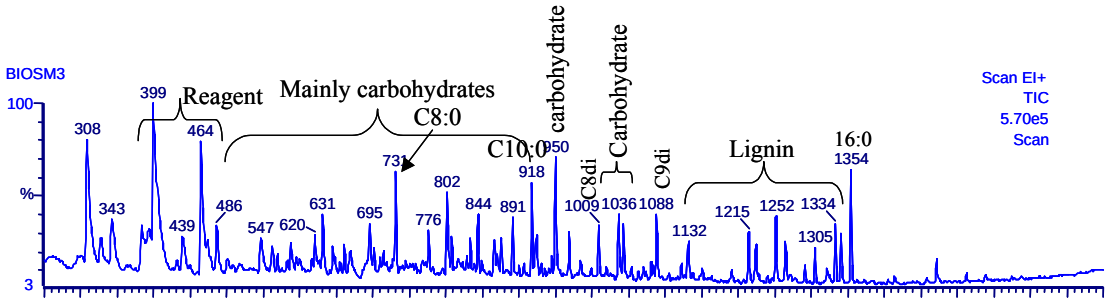

Figure 6. Chromatogram obtained by py-GC-MS analysis of the hot-water extract originating from sample BSP 1. The chromatogram demonstrates several peaks from pyrolysis products originating from water-soluble carbohydrates. Only a few lignin-derived pyrolysis products were found in this chromatogram.

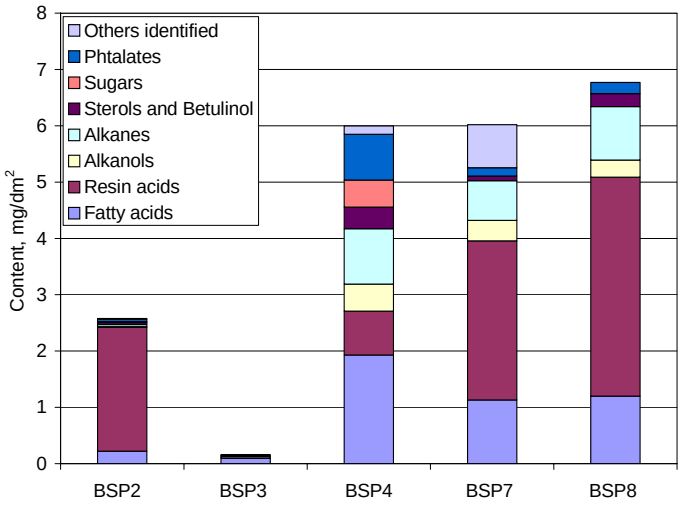

Figure 7. Contents of different type of compounds in 95% ethanol extracts obtained by extraction of paper samples using the total immersion test procedure (mass of the compounds per surface area of the sample intended for food contact, mg/dm^2). The results documented in the diagram were obtained by calculating, for each class of extractive, the sum of the individual compounds identified and quantified by the GC-MS analysis.

The identified organic compounds (extracted with 95% ethanol employing the total immersion procedure) were also classified into groups of different types of extractives, i.e.; fatty acids, resin acids, sterols and betulinols, sugars, alkanes and alkanols, phthalates and other identified compounds. As can be seen from Figure 7., the ethanol extract obtained from sample BSP 2 was composed mainly of resin acids that probably originated from rosin sizes. The ethanol extracts obtained from the samples BSP 7 and BSP 8 also contained relatively large portions of resin acids according to the data in reported the diagram.

5.3.1.2. NSP Series

Dry matter content of the water extracts

The dry matter content for the six water extracts, NSP6, 7, 11 and 18-20, is reported in Table W1. In the case for the five cold water extracts (NSP6, 7, and 18-20), the dry matter content (DMC) varied from 48 mg/L and up to 312 mg/L. The quantity of recovered cold water extractable matter ranged from 0.8 up to 18.7 mg/dm^2 calculated on basis of paper/board surface area intended for wet food contact. Moreover, the dry matter content of the hot water extract, NSP18, was 472 mg/L. Thus, the quantity of recovered hot water extractable matter was 23.1 mg/dm^2 calculated on basis of paper/board surface area intended for wet elevated temperature food contact.

GC-MS identification of the substances in the water extracts

Many different substances were identified by GC-MS analysis of the derivatized water extracts from the four paper/board samples NSP6, NSP7, NSP19 and NSP20. In contrast, only a few substances were identified in the derivatized water extracts from NSP11 and NSP18 (see Figure 8). Table 15 reports the quantity of identified substances summarize into different types of compound classes as well as the total sum of substances quantified. The quantity and identity of the individual substances identified in each of the extracts are reported in the Tables W3-8 (Annexes). The identification of a detected substance was done by comparison of its mass spectrum recorded with the corresponding library mass spectrum present in our MS-library data base. The best library match found for each identified substance is reported in the tables. In certain case, e.g., for many of the fatty and resin acids, the library match identifications were also confirmed by separate GC-MS analysis of the actual compounds or mixtures thereof. The estimated concentrations given in Tables

W2-8 (Annexes) were obtained 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.

Table15. Sample number, recycled fibre content, test conditions, grammage and content of extractable matter for the nominated paper and board (NSP) samples extracted by STFI-Packforsk.

Sample Number	**Recycled fiber content**	**Simulant, t/T test conditions**	**Grammage[a]** (g/m^2)	**Grammage[b]** (g/m^2)	**Extractable matter** (mg/g)	**Extractable matter** (mg/dm^2)
NSP 6	98 %	Water Time:24 h, Temp:20 °C	300	280	6.1	17.1
NSP 7	0 %	Water Time:24 h, Temp:20 °C	285	272	1.2	3.2
NSP 11	0 %	Water Time:2 h, Temp:80 °C	210	196	11.8	23.1
NSP 18	0 %	Water Time:24 h, Temp:20 °C	240	228	7.8	18.7
NSP 19	0 %	Water Time:24 h, Temp:20 °C	300	290	3.3	9.6
NSP 20	80 %	Water Time:24 h, Temp:20 °C	30	28	2.8	0.8
NSP 4	100%	Water Time:24 h, Temp:20 °C	-	281	3.3	9.2

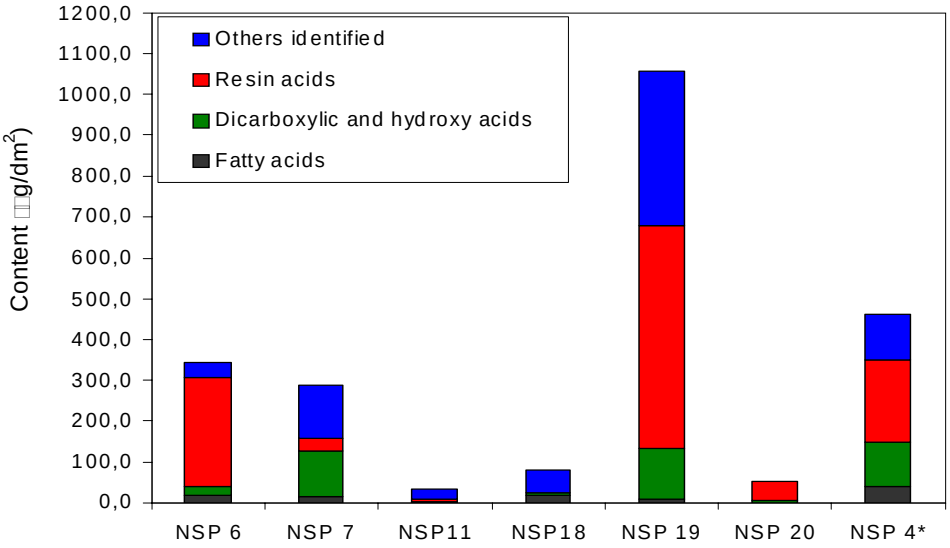

Figure 8. Quantities of the different compound classes detected in the water extracts from the nominated paper and board samples NSP 6, NSP 7, NSP 11, NSP 18 NSP 19 NSP 20 and NSP 4* (supplement sample). The analyses were performed by gas chromatography and mass spectrometry on derivatised (silylated) water extracts.

NSP6 - Table W3 reports the individual substances identified in this cold water extract. Two substances were found in excess of 50 µg/dm^2, dehydroabietic acid 152 µg/dm^2) and abietic acid (55 µg/dm^2). In addition, three other resin acid isomers (15, 17 and 12 µg/dm^2) and a sugar alditol (10 µg/dm^2) were found within the quantification range 10-50 µg/dm^2. Table W2 reports the total quantity of resin acids detected in this water extract, as well as, the quantities of fatty acids, dicarboxylic/ hydroxy acids and other identified compounds. It is worth noting that total quantity of resin acids accounted for the major part, ~ 80 %, of the whole GS-MS identifiable extract fraction. However, the total quantity of GC-MS detected compounds (345 µg/dm^2) accounted only for a minor fraction, ~2 %, of the total dry matter content determined gravimetrically (17100 µg/dm^2).

NSP7 – In the case for this extract, no substances were found above the 50 µg/dm^2 quantification level, see Table W4. However, a wide range of different types of substances were detected within the range 10-50 µg/dm^2, glycerol (37 µg/dm^2), boric acid (27 µg/dm^2), hydroxyacetic acid (24 µg/dm^2), 3,4-di-hydroxy butanoic 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). Table W2 reports the total quantity of different classes of identified compounds. In

this case, the total quantity of GC-MS detected compounds (290 μg/dm^2) constituted ~9 % of the dry matter content (3200 μg/dm^2).

NSP11 – No substances were found above the 50 μg/dm^2 level, see Table W5. Only two substances were found in the range 10-50 μg/dm^2, methylsiloxane oligomer (15 μg/dm^2) and ethyleneglycol (12 μg/dm^2). The total quantity of GC-MS detected compounds (37 μg/dm^2) constituted a very small fraction, < 0.2 %, of the total dry matter content (23100 μg/dm^2) of this extract, see Table W2

NSP18 – No substances were found above the 50 μg/dm^2 level, see Table W6. Three substances were found within the range 10-50 μg/dm^2, ethyleneglycol (46 μg/dm^2), methylsiloxane oligomer (11 μg/dm^2) and oleic acid (11 μg/dm^2). Also in this case, the total quantity of GC-MS detected compounds (80 μg/dm^2) constituted a very small fraction, ~0.4 %, of the dry matter content (18700 μg/dm^2) of the extract, see Table W2.

NSP19 – Several substances were found above the 50 μg/dm^2 quantitation level, boric acid (197 μg/dm^2), abietic acid (168 μg/dm^2), dehydroabietic acid (159 μg/dm^2), two resin acid isomers (82 and 58 μg/dm^2), triethyleneglycol (54 μg/dm^2) and glycerol (52 μg/dm^2). In addition, a number of different types of substances, including several resin acids, were detected within the range 10-50 μg/dm^2 and reported in Table W7. In this case the total quantity of resin acids constituted more than 50 % of the whole GS-MS identifiable extract fraction. Moreover, the total quantity of GC-MS detected compounds (1055 μg/dm^2) accounted for more than 10 % of the total dry matter content (9600 μg/dm^2), which is the highest figure obtained among all the investigated water extracts (see TableW2).

NSP20 – No substances were found above the 50 μg/dm^2 level, see Table W8. Within the range 10-50 μg/dm^2 two resin acids were found, dehydroabietic acid (20 μg/dm^2) and abietic acid (12 μg/dm^2). In this case, the total quantity of GC-MS detected compounds (54 μg/dm^2) constituted a very small fraction, 6.8 %, of the dry matter content (800 μg/dm^2), see Table W2.

*Chemical characteristics of the ethanol extracts (**Tables 17 - 34 are attached as appendices)***

Dry matter content of the ethanol extracts

The DMC of the plain ethanol extracts are shown in Table 16. The results are expressed in mg per litre of solvent used and also as mg per kg of paper/board extracted. Since the ethanol extracts were supplied to Module 1 partners after a 10-fold concentration by evaporation, the DMC in the extracts supplied would be 10-times the values shown in Table 16, and in the range 1340 to 7070 mg/L.

Table 16. Characteristics of the plain ethanol extracts

	NSP4	**NSP5**	**NSP8**	**NSP13**
Volume recovered	1200 mL	1200 mL	1800 mL	1800 mL
Dry matter content *	519 mg/L	707 mg/L	134 mg/L	380 mg/L
DMC (mg residue per kg paper)	5190 mg/kg	7070 mg/kg	1340 mg/kg	3800 mg/kg

*since the ethanol extracts supplied for toxicity assays were concentrated 10-fold, the DMC as tested are 10-times these values

GC-MS Identification of the substances in the concentrated ethanol extracts

(Results are shown in Tables 17-34 as Annexes)

A number of substances were detected in the concentrated ethanol extracts of paper/board samples NSP4, NSP5, NSP8 and NSP13. Their identities proposed by comparison with library spectra and their estimated concentrations (calculated relative to the nearest internal standard) are shown in Tables 17 to 20. The identities proposed are the best library match but have not been confirmed through the analysis of standard compounds. The estimated concentrations given in Tables 17 to 20 have been estimated by comparing the peak area of the substance present with that of the nearest internal standard and therefore assumes uniform response factors.

NSP4 - The substances present in excess of 50 μg/dm^2 were (Table 15) the DIPN isomers (total 650 μg/dm^2), diisobutyl and dibutyl phthalate (360 and 110 μg/dm^2), bis(2-ethylhexyl) phthalate (89 μg/dm^2), 9-octadecenoic acid (62 μg/dm^2), dehydroabietic acid (110 μg/dm^2) and a number of

straight chain alkanes (52, 53, 54 and 100 μg/dm^2). Additional substances were detected below this level of 50 μg/dm^2. These are listed in Table 17, but not described in the commentary here.

NSP5 - The substances present in excess of 50 μg/dm^2 were 1-methyl-2-pyrolidinone (1700 μg/dm^2), 2-phenylphenol (53 μg/dm^2), the DIPN isomers (total 210 mg/dm^2), diisobutyl and dibutyl phthalate (370 and 58 mg/dm^2), 1-cyclohexene-1-carboxylic acid, 4-(1,5-dimethyl-3-oxohexyl)-, methyl ester [S-(R*,R*)]- (180 μg/dm^2), 9-octadecenoic acid (160 μg/dm^2), bis(2-ethylhexyl) fumarate (63 μg/dm^2), eicosene (280 μg/dm^2), methyl dehydroabietate (77 μg/dm^2), dehydroabietic acid (120 μg/dm^2), bis(2-ethylhexyl) phthalate (120 μg/dm^2), stigmast-7-en-3-ol, (3.beta.,5.alpha.,24S)- (64 μg/dm^2), a number of straight chain alkanes (58, 70, 66, 83 and 130 μg/dm^2) and two substances for which no good library match was obtained (280 and 470 μg/dm^2). Additional substances were detected below this level of 50 μg/dm^2. These are listed in Table 18. but not described in the commentary here. A number of additional substances were detected in the ethanol extract of NSP5 which, although no good library match was obtained, showed mass spectra characteristic of perfluorinated greaseproofing agents and their breakdown products.

NSP8 - Only low levels of substances were detected in the extracts. The highest concentration estimated was for stigmast-7-en-3-ol, (3.beta.,5.alpha.,24S)- present at 47 μg/dm^2. The levels and identities of the other substances detected are shown in Table 19.

NSP13 - Vanillin (93 μg/dm^2), diisobutyl phthalate (99 μg/dm^2), 1-octadecene (210 μg/dm^2), 9-octadecenoic acid (130 μg/dm^2), octadecanoic acid (73 μg/dm^2), eicosene (99 μg/dm^2), stigmast-7-en-3-ol, (3.beta.,5.alpha.,24S)- (120 μg/dm^2) and two substances for which no good library match was obtained (64 and 370 μg/dm^2). Additional substances were detected below this level of 50 μg/dm^2. These are listed in Table 20. but not described in the commentary here.

<u>GC-MS identification of the substances in the CEN ethanol extracts</u>

CEN extract of NSP4 - As expected, the levels of substances extracted from NSP4 using the 'concentrated' procedure (Table 17) and using the standard CEN procedure (Table 21) are similar

when expressed on a surface area basis but of course the CEN extracts were much more dilute. When comparing the data there were some differences in retention time due to a different GC-MS system being used for the analysis. Some slight differences do exist, for example the concentration of dibutyl phthalate (110 versus 83 μg/dm^2) but in the context of this work where substances were estimated using the assumption that response factor compared to internal standard was unity and the individual substances were not used to construct calibration graphs, such a difference is not consequential. The major substances were detected in both extracts. The general agreement between the two types of extracts give reassurance that a) the concentrated procedure using a large quantity of paper/board does not give rise to solubility limitations; b) the evaporation procedure used for concentration, does not give rise to unacceptable losses of substances.

The CEN extract had fewer substances tabulated simply because these fell below the detection limit for the more dilute extract. Tetradecane, benzyl benzoate and isopropyl dodecanoate were detected in the 'CEN' extracts but were not reported as being present in the concentrated extract. Re-examination of the chromatogram of the concentrated extract confirmed that all three were present but that they co-eluted with other extractables or interfering substances which masked their mass spectra.

CEN extract of NSP8 - No substances were detected in the CEN extracts. The limit of detection based on 3 x the signal: noise ratio varied with the analyte retention time but in the worst case was 1.5 μg/ml which is equivalent to 77 μg/dm^2 in the paper/board sample. No substances were detected in the concentrated extracts of this sample (Table 19) at levels in excess of 77 μg/dm^2.

CEN extract of NSP13 - A number of substances were detected in the CEN extract of sample NSP13 and they are listed in Table 22. Again as expected, the levels expressed on an area basis were similar to those for NSP13 reported in Table 20.

Derivatised extracts

The results above were obtained from the GC-MS analysis of the ethanol extracts without derivatisation. With silylation, a number of trimethylsilyl esters were detected for the four samples. Their identities were consistent with esters of those acids already detected in the direct analysis of the concentrated ethanol extracts. No additional information was obtained from the derivatisation studies.

Mass balance: GC-MS results versus dry matter content

The sum of all substances estimated in the GC-MS analysis (Tables 17-20) can be compared with the gravimetric dry matter content obtained by evaporation of the concentrated ethanol extracts (Table 16). The results are as follows (identity; DMC in mg/kg; sum GC-MS in mg/kg, GC-MS as percentage). NSP4, 5190, 757, 15%. NSP5, 7070, 899, 13%. NSP8, 1340, 81, 6%. NSP13, 3800, 224, 6%. This reveals that only a small proportion, 6 to 15%) of the extractable matter can be detected and estimated using GC-MS. Even some of the GC-MS peaks that were detected could not be identified clearly. This helps to illustrate the need for a global assessment of the safety of the total migrate from paper and board, which chemical analysis alone cannot provide.

Identification of substances in a low molecular weight fraction of the ethanol extract of NSP4

The LMWF of NSP4 contained 133 mg/L DMC or 6.65 mg DM for the 50 mL fraction collected. Since this fraction originated from 0.0482 kg paper (0.188 x 0.5/1.95) the LMW DMC was 138 mg/kg. The unfractionated DMC for NSP4 was 5190 mg/kg (Table 16) so the LMWF after drying contained just 2.7% of the extractable DMC.

However, a quite different conclusion can be drawn from the GC-MS results. The relevant tables are Table 17 (whole extract) and Table 23 (LMWF). First, it is remarkable that the sum of all GC-MS peaks detected are very similar, at 2.27 versus 2.32 mg/dm^2. Second, it is clear that the LMWF has picked-up some extraneous siloxanes and BPA most probably from the materials used in the membrane fractionation apparatus or the membrane itself. Third, the LMWF has lost some of the minor hydrocarbons (pentadecane, hexadecane, nonadecane, tricosane, tetracosane etc) 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. Fourth, the LMWF is 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 matters of detail notwithstanding, it is remarkable that not only is the total concentration of GC-MS substances comparable (Tables 17 and 23) but also the concentration of many indicator substances are quite similar. For example (substance abbreviation, whole fraction v. LMWF in

ug/dm^2). DIPN, 660 v. 600. DEHP, 89 v. 120. TMB, 81 v. 82. DBP, 110 v. 64. DiBP, 360 v. 310.

The final point to note is that the sum of GC-MS results is 764 mg/L (Table 23) whereas the DMC measured after evaporation of this sample was only 133 mg/L (see above). This reveals 2 things. a) the fractionation 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. In conclusion, the membrane fractionation procedure is effective in isolating the LMWF although it has changed the detailed profile of the extract due to release and absorptive effects of the equipment used. Further concentration of the fraction for toxicity testing, by evaporation, would be hindered by volatilisation losses unless an inert 'keeper' was used.

Chemical characteristics of the Tenax extracts

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

GC-MS identification of the substances in the Tenax extracts

A number of substances were detected in the ethanol extracts of the Tenax exposed to paper/board samples NSP1, NSP2, NSP3, NSP9, NSP10, NSP12, NSP14, NSP15, NSP16 and NSP17. Their identities proposed by comparison with library spectra and their estimated concentrations (calculated relative to the nearest internal standard) are shown in Tables 24 to 33. The identities proposed are the best library match but have not been confirmed through the analysis of standard compounds. The estimated concentrations given in Tables 24 to 33 have been calculated by comparing the peak area of the substance present with that of the nearest internal standard and therefore assumes that they have the same response factor.

Although a number of substances were found to migrate into Tenax the levels were much lower than for the ethanol extracted samples. Only NSP2 (DIPN isomers 140 µg/dm^2 and diisobutyl phthalate 110 µg/dm^2), NSP10 (DIPN isomers 120 µg/dm^2 and diisobutyl phthalate 61 µg/dm^2), NSP12 (diisobutyl phthalate 50 µg/dm^2 and bis(2-ethylhexyl) phthalate 66 µg/dm^2) and NSP14

(DIPN isomers 110 μg/dm^2 and diisobutyl phthalate 98 μg/dm^2) gave rise to migration in excess of 50 μg/dm^2.

In preparatory work using the BSP-series of samples, it was found that for the migrate into Tenax, derivatisation of the extract did not provide any significant extra information. This was not surprising because, as stated above, migration into this dry powdered polymer occurs mainly through the gas phase. Consequently it can be expected that the migrate will be amenable to direct GC-MS analysis since this is a vapour phase technique and derivatisation to make more volatile is not needed.

Additional tests using Tenax with Sample NSP4

Based on the initial toxicity tests results on the ethanol extract of sample NSP4 (a non-food grade paper sample included in this project as a worst case) it was considered of interest to compare the ethanol extract with a Tenax extract. The Tenax extract in an ethanol vehicle was prepared in the standard way and when sent to the Module I partners the extract was coded T1. The substances detected by GC-MS are listed in Table 34.

5.3.2. Toxicity tests on model paper and board extracts (BSP series)

The results of the different assays performed on the extracts of some of the BSP samples (not all BSP samples were tested) are summarised in Table 35. The extracts had been prepared according to the standard procedures without any attempts to concentrate the extractants before testing. It can be seen that the samples were mostly negative, only marginal signs of toxicity could be seen in some samples. The results of Bioluminescence test were particularly difficult to interprete, since most of the samples marked as negative in the table actually caused a stimulation of bioluminiscence (data not shown).

Table 35. The results of the cytotoxicity tests on extracts of BSP samples (for colour codes, see Table 11)

Samples*		Maximum concentration in the test	Assay				
			V. fischeri test	Boar spermatozoan motility assay	RNA-synthesis inhibition test (HepG2 cells)	Acute cytotoxicity tests	
						Hepa-1 cells	HEp-2 cells
Ethanol extract of BSP7 (recycled chipboard)		2 %					
Ethanol extract of BSP9 (SBS bleached kraft)		2 %					
Hot water extract of BSP1 (tea filter)		80 - 90%					
Cold water extract of BSP5 (test liner)		80 - 90%					
Fractionated water extract of BSP 6 (fluting, semi chemical)	MW < 1000	80 - 90%					
	MW > 1000	80 - 90%					
Fractionated water extract of BSP7 (Chipboard)	MW < 1000	80 - 90%					
	MW > 1000	80 - 90%					

* For the dry matter content of the extracts, see Tables 13 and 14

5.3.3. Tests on spiked paper and board samples and extracts

Paper spiked with B. cereus emetic toxin

Ethanol extract of spiked paper was strongly toxic in all the cytotoxicity assays indicating that significant amounts of cereulide were leached into the extract (Table 36.). On the contrary, neither cold or hot water extracts of spiked paper caused any positive response in the tests (the pentane extract of the hot water extract showed some toxicity in the boar spermatozoan motility inhibition assay. Data not shown).

Table 36. The responses of the different cytotoxicity assays to ethanol and water extracts of paper spiked with cereulide.

	Boar spermatozoan motility assay (sample at 2% concentration)	Tests on cultured mammamlian cells			
		Maximum tested concentration	RNA-synthesis inhibition test (HepG2 cells)	Acute cytoxicity tests	
				Hepa-1 cells	Hep-2 cells
Ethanol extract		1 - 2%			
Cold water extract		80 - 90%			
Hot water extract		80 -90%			

= sample caused either a total cell death or inhibition of RNA synthesis at the top tested dose

= no indication of toxicity even at the highest doses tested

Acrylamide spiked extracts

The spiking of paper and board extracts with acrylamide did not produce any major effect on the toxicity of the compound in most of the tests (Table 37). Some synergistic action could be seen in boar spermatozoan motility inhibition assay with BSP9 ethanol extract. Generally, the differences probably reflect the normal fluctuation of biological test systems rather than actual different response.

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

Table 37. The toxicities of acrylamide-spiked paper and board extracts

Solvent	Laboratory	The EC_{50} of acrylamide dissolved in paper and board extracts			Inhibitory concentration of acrylamide dissolved in paper and board extracts
		Cytotoxicity (NRU, 24h) in HEp-2 cells	Cytotoxicity (TPC, 24h or 72h) in Hepa-1 cells	RNA-synthesis inhibition in HepG2 cells	Boar spermatozoan motility inhibition assay (72h)
water	ISS	6,4 mM[a]			
	UKU		9,4 mM (24h)[b] 3,5 mM (72h)[b]		Non-inhibitory at 1,6 mM[c]
	ENSBANA			4.09 mM	
water extract of BSP5	ISS	9,6 mM[a]			
	UKU		6,0 mM (24h)[b] 3,1 mM (72h)[b]		Non-inhibitory at 1,6 mM[c]
	ENSBANA			3.98 mM	
Ethanol 1%	ISS	6,4 mM			
	UKU		6,2 mM (24h) 2,0 mM (72h)		20,1 mM
	ENSBANA			3.27 mM	
ethanol extract of BSP9 1%	ISS	4,8 mM			
	UKU		6,5 mM (24h) 2,3 mM (72h)		5,1mM
	ENSBANA			3.98 mM	

[a] "water" or "water extract of BSP5" content 5% .
[b] "water" or "water extract of BSP5" content 90% in cytotoxicity (TPC) assay in Hepa-1 cells.
[c] "water" or "water extract of BSP5" content 8% in boar spermatozoan motility inhibition assay.

B[a]P spiked extracts

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

A dose-dependent increase of the genotoxic effect due to B[a]P was observed. The effect was not influenced by the solvent (NSP4 extract versus ethanol or NSP11 extract versus water). However, the effect of DiPNs was variable. With NSP4, DiPNs did not influence the results at the lowest concentration of B[a]P (1 µM), but a decrease of the genotoxicity appeared in cells treated with DiPNs together with 5 or 10 µM of B[a]P (Figure 9.). With NSP11, a slight increase in genotoxicity appeared at 6.25 µM B[a]P when tested together with DiPNs, but there was no effect at lower B[a]P concentrations (Figure 10.).

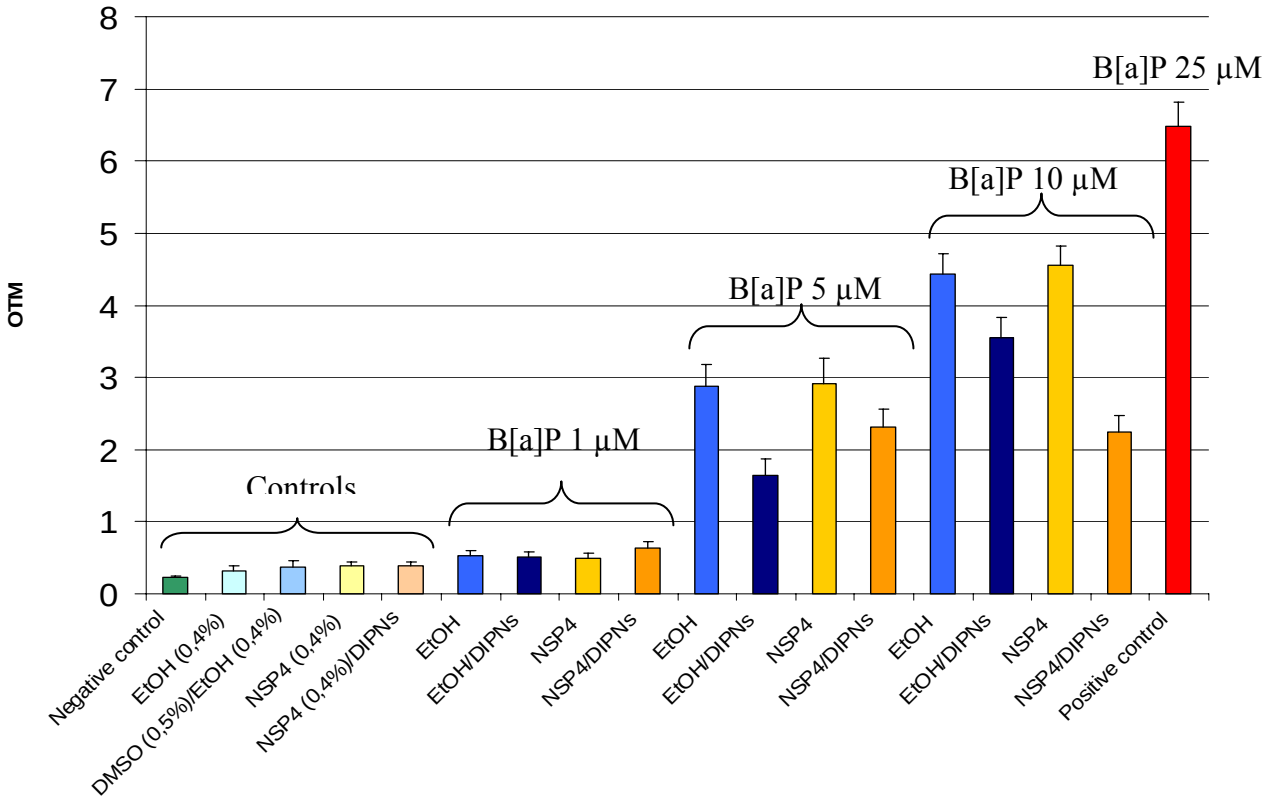

Figure 9. The interaction of DiPNs on genotoxic effect of benzo(a)pyrene (B[a]P) spiked ethanol extract. of NSP4 in comet assay.

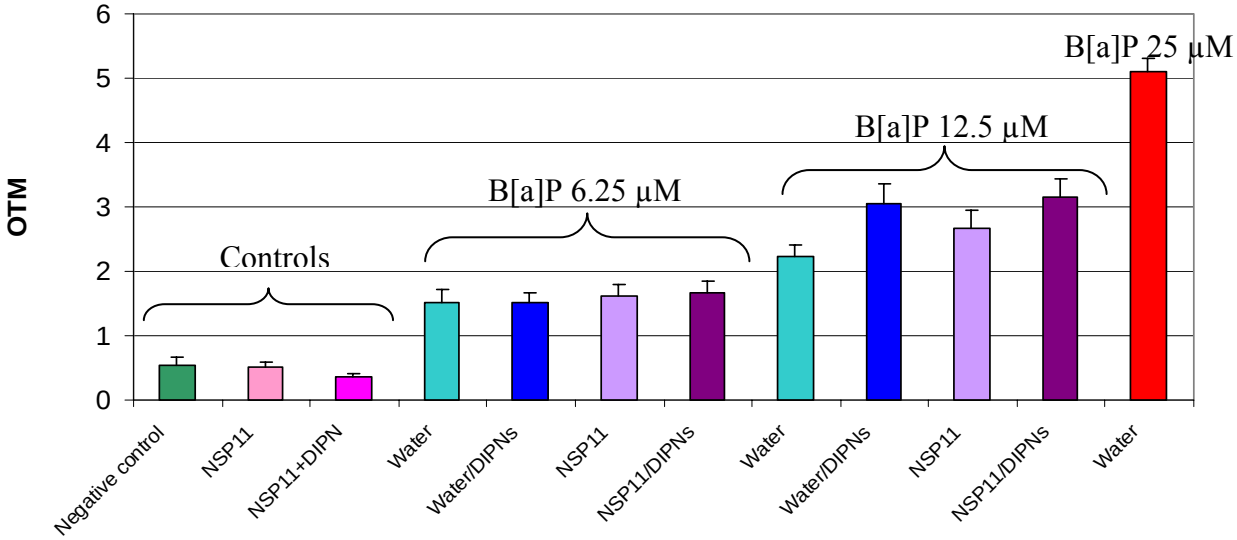

Figure 10. The interaction of DiPNs on genotoxic effect of benzo(a)pyrene (B[a]P) spiked water extract. of NSP11 in comet assay.

In conclusion, the competitive action of DiPNs on the xenobiotic metabolising pathways (CYP450) was not observable at low concentrations of B[a]P, but may decrease the genotoxicity at higher B[a]P concentrations, possibly by saturating the metabolic activation pathways.

5.4. Tests on actual paper and board samples provided by industry (NSP series)

5.4.1. Selection of the final cellular toxicity tests and genotoxicity assays

The tests considered in the beginning of the project are presented in Figure 11. In the subsequent assessment there was, 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 were selected as the basis of the final test battery of the cellular toxicity.

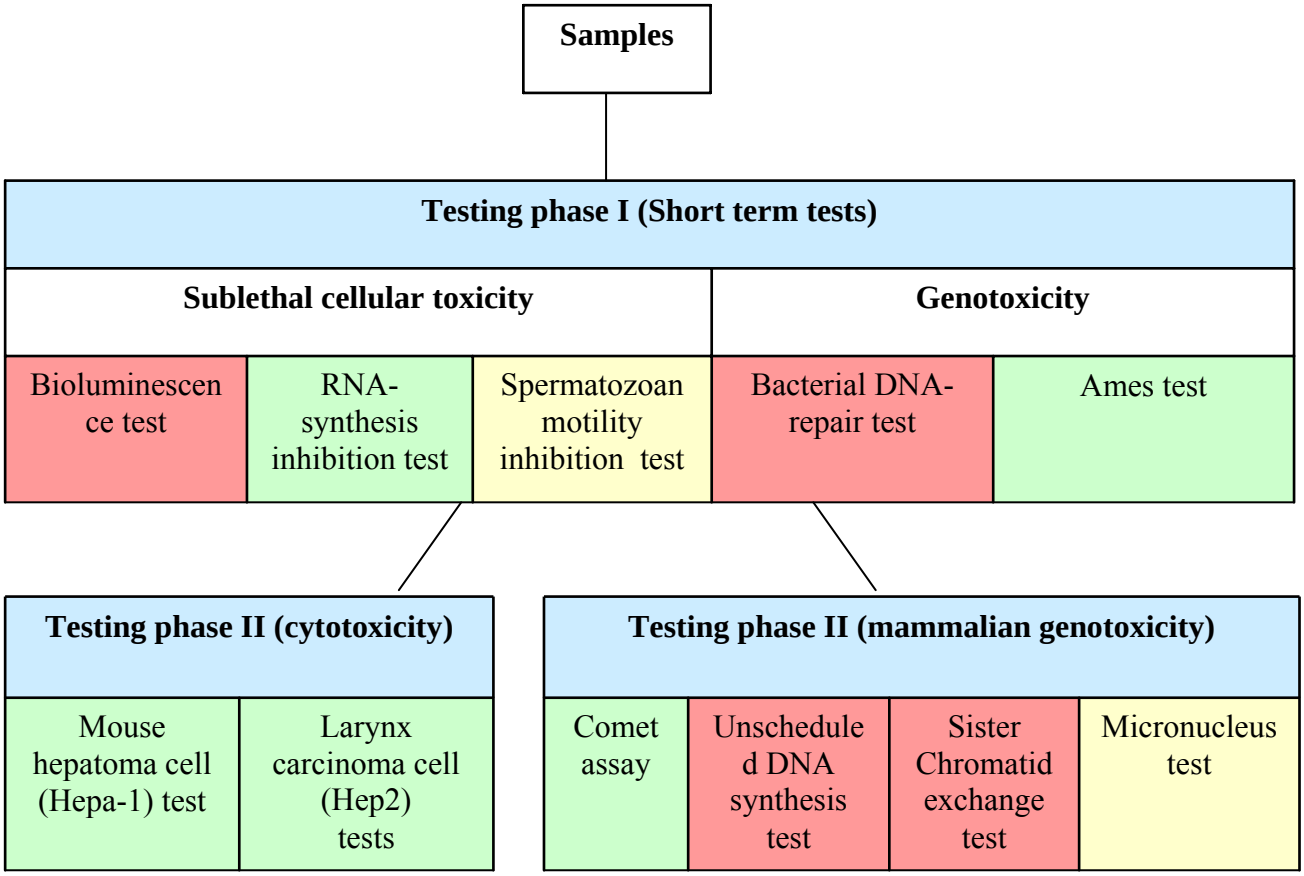

Figure 11. The tests originally considered for the project. The tests selected for the final test battery are marked green, the ones that can be used as optional complementary tests are marked yellow, and those that were rejected during the project are marked red. (the testing phases indicated in the original scheme do not correspond the final decision-tree approach depicted in Figure 12).

The boar spermatozoan motility inhibition is a highly specific test, that can be used to complement the basic test battery. The usefulness of the bioluminescence test was considered limited, because the endpoint is sometimes difficult to interpret (the fluctuating stimulation and inhibition of bioluminescense during the 30 minute measuring time). Regarding the genotoxicity assays, the Ames test was included as a matter of course to test the eventual mutagenic activity. Of the mammalian genotoxicity assays the Comet assay was chosen, since there was a good agreement between the results of the different performing laboratories in tests with model compounds. On the basis of the experience gained during the project, the following tests (coloured green in the scheme above) were chosen from the original selection of tests considered for the project: Acute cytotoxicity tests with hepa-1 and HEp-2 cells, the RNA-synthesis inhibition assay, the boar spermatozoan motility inhibition assay, Ames test and comet assay.

5.4.2. Results of the NSP series

The 20 project samples selected by industry and extracted by MAFF-CSL and STFI and designated by NSP ("Nominated Samples for Project") were tested by the project partners ENSBANA, ISS, NPHI, UH.DACM and UKU. The summarized results are given in Tables 38, 39 and 40.

Table 38. Results of the paper and board samples representing contact with dry foods and extracted with a modified Tenax-procedure. The maximum amount of final ethanol extract in the cytotoxicity tests was 1-2% (For colour codes, see Table 11)

Sample*	Acute cytotoxicity		RNA-synthesis inhibition		Boar spermatozoan motility inhibition	Genotoxicity	
	HEp-2 cells	Hepa-1 cells	HepG2-cells	Hela cells		Ames test	Comet assay
NSP1							
NSP2							
NSP3							
NSP9							
NSP10							
NSP12							
NSP14							
NSP16							
NSP17							

* For the dry matter content of the sample extracts see Tables 24 - 32.

Table 39. Results of the paper and board samples representing contact with wet foods and water extracted using the standard CEN procedure. The maximum amount of water extract in the cytoxicity tests was 80 -90 %, or 2 % in the boar spermatozoan motility inhibition assay (For colour codes, see Table 11)

Sample*	Acute cytotoxicity		RNA-synthesis inhibition		Boar spermatozoan motility inhibition	Genotoxicity	
	HEp-2 cells	Hepa1 cells	HepG2-cells	Hela cells		Ames test	Comet assay
NSP6							
NSP7							
NSP11							
NSP18							
NSP19							
NSP20							

* For the dry matter content of the sample extracts, see Table 15

Table 40. 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. (for colour codes, see Table 11)

Sample*	Acute cytotoxicity (the highest tested conc. 2%)		RNA-synthesis inhibition (the highest tested conc.2% for HepG2, 0.5 % for Hela cells)		Boar sperm. motility inhibition (the highest tested conc. 2%)	Genotoxicity	
	HEp-2 cells	Hepa-1 cells	HepG2-cells	Hela cells		Ames test	Comet assay
NSP4	EC_{50} 0.5 ± 0.1	EC_{50} 0.44 ± 0.03	EC_{50} 0.38 ± 0.03	synthesis 16 ±7 % of the control	the lowest toxic dilution 0.25%	strain TA98 without S9	
NSP5	EC_{50} 0.6± 0.5	EC_{50} 0.45 ± 0.01	EC_{50} 0.29 ± 0.02	synthesis 9 ±1 % of the control	the lowest toxic dilution 0.25%		
NSP8	EC_{20} 1.0± 0.2	EC_{20} 2.0 ± 0.01	EC_{50} 1.49 ± 0.12	synthesis 55± 3% of the control	Only the top concentration toxic		
NSP13	EC_{20} 0.8± 0.4	EC_{50} 1.90 ± 0.1	EC_{50} 0.92 ± 0.01	synthesi 34 ± 3% of the control	The highest toxic dilution 0.5%		

***** For the dry matter content of the extracts, see Tables 17 -19

As can be seen, the only samples showing consistent positive responses were the ethanol extracts of NSP4, NSP5, NSP8 and NSP13. Moreover, NSP4 was also the only sample giving an indication of genotoxic activity. The actual Ames test results of this sample are given in Table 41. It can be seen that the effect was specific to the tester strain TA98 without the metabolic activation system.

Table 41. The response of the Ames tester strains TA100, TA98 and TA97 to the concentrated ethanol extract of NSP4

NSP4 (ethanol extract), dose per plate (µl)	**Revertants per plate (mean ± sd of three plates)**					
	TA100		**TA98***		**TA97**	
	-S9	**+S9**	**-S9**	**+S9**	**-S9**	**+ S9**
200	116 ± 2.6	100 ± 4.4	**92 ± 8.0***	**43 ± 6.4**	108 ± 7.6	85 ± 12.2
100	118 ± 15.3	164 ± 7.8	73 ±14.4		126 ± 17.6	143 ± 10.4
			90 ± 12.9	**27 ± 7.5**		
50	140 ± 11.2	166 ± 3.4	45 ± 4.6		119 ± 12.3	151 ± 15.6
			31 ± 9.7	**40 ± 8.9**		
25	138 ± 5.0	150 ± 7.1	41 ± 6.0			
10	128 ± 5.0	157 ± 1.6	33 ± 2.1			
5	137 ± 9.3	168 ± 6.8	26 ± 5.9			
0	129 ± 7.8	143 ± 7.2	27 ± 11.0		102 ± 8.5	121 ± 7.2
			26 ± 3.2	**57 ± 11.6**		

* The test was repeated after the first preliminary test (**in bold)** using more concentrations to confirm the dose-response.

After the results from tests with concentrated ethanol extracts of samples NSP4, NSP5, NSP8 and NSP13 had been obtained, the Hepa-1 cytotoxicity and RNA-synthesis inhibition tests tests were repeated using the standard (non-concentrated) ethanol extracts of samples NSP4, NSP8 and NSP13, the corresponding Tenax extracts and a standard cold-water extract of NSP4. The results of the cytotoxicity assays were negative (data not shown). With NSP4 also Ames test was performed. with negative results (data not shown).

5.5. Implementation on risk assessment

On the basis of the accumulated results both on model compounds and actual paper and board samples the following proposal for a decision tree was formulated (Figure 12). This approach proposes a rationale for **hazard identification,** which then can be used as a basis of actual **risk assessment.** The different stages of the process are given in the following chapters.

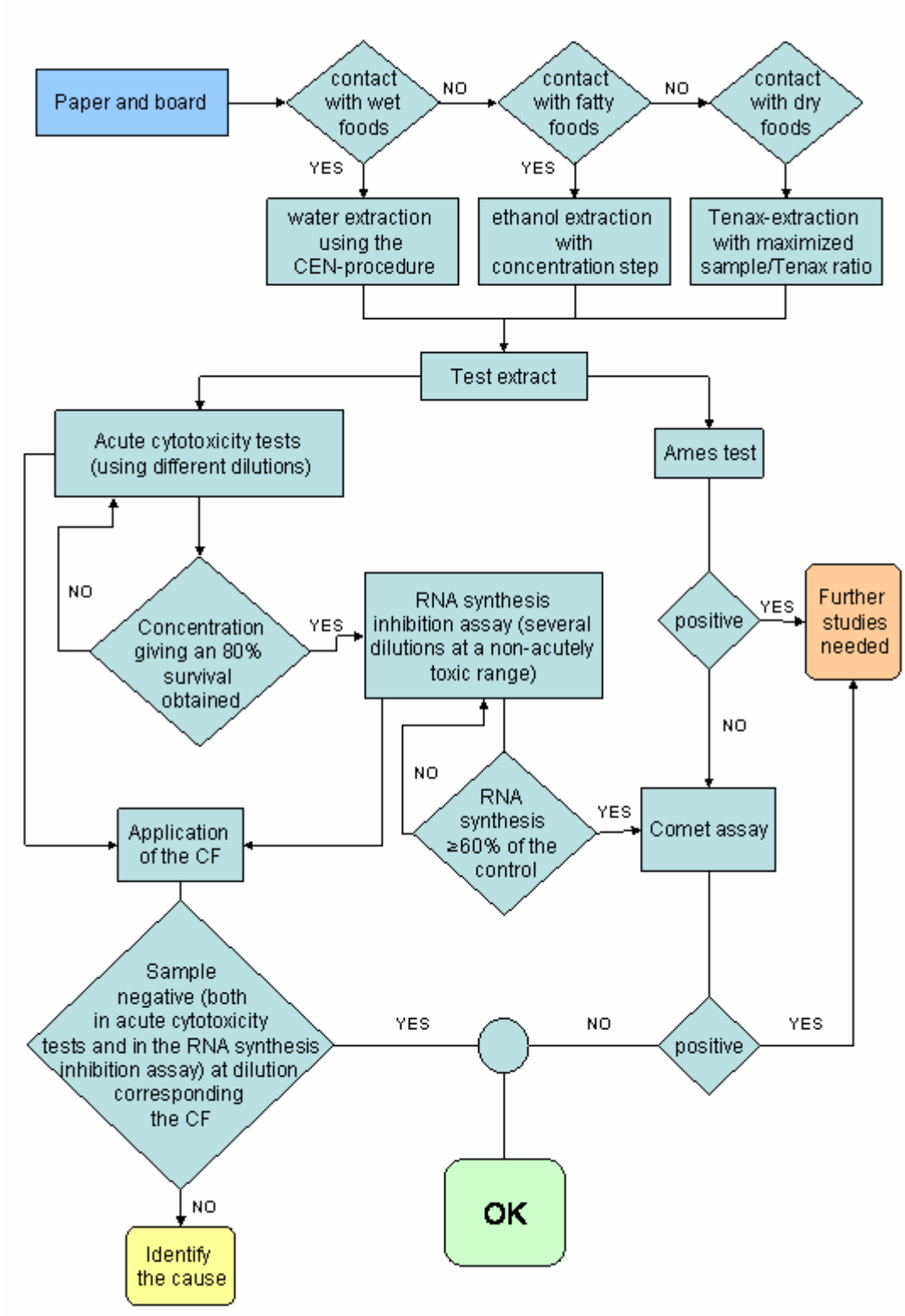

Figure 12. The decision tree based test battery for hazard identification based on the experience gained during the project

5.5.1. Paper and board samples

The test extract is prepared according to the procedures described in Module II of the project. The aim is to expose the biological test systems to maximal amounts of extractables. In case of water extracts (simulating wet or moist foods) this can be done by reconstituting the biological test systems in the extract itself. For ethanol extracts (simulating fatty foods) or ethanol extracts of Tenax (simulating dry foods) one has to take into account the low tolerance of the biological test systems to ethanol. So the ethanol solution is prepared in a more concentrated form to compensate for the dilution effect when it is added in small amounts to the biological test systems. The end result is that the biological test systems are exposed to a comparable quantity of paper/board equivalents whether water or ethanol solutions are tested.

5.5.2. The actual test battery

Two aspects of toxic responses are measured: genotoxicity and cytotoxicity.

- Genotoxicity or the ability of the sample to interfere with the genetic machinery is tested using the Ames test, which is a validated and well established test for point mutations, and with comet assay, which measures physical DNA-damage in mammalian cells. Comet assay. is also the test in which the cytotoxicity and genotoxicity assays interact, since it is necessary to use non-cytotoxic concentrations in order to avoid false positives. **Therefore cytotoxicity assays are a necessary preparatory step for the comet assay in addition to providing information on the cytotoxic potential of the sample.**
- The cytotoxic potential is tested using both acute cytotoxicity assays, in which cell death is the end point, and the RNA-synthesis inhibition test, which measures the sublethal effects. These tests are mutually complementary, since, in order to have meaningful results in the RNA-synthesis inhibition assay the tested concentrations should be not lethal to the cells. **It should be noted that in both types of cytotoxicity assays different dilutions of the test agent are used in order to get a dose-response curve and to get an indication of the types of food contact applications for which the paper/board product would be suitable for after application of the relevant Correction Factor (CF) (Figure 13)**

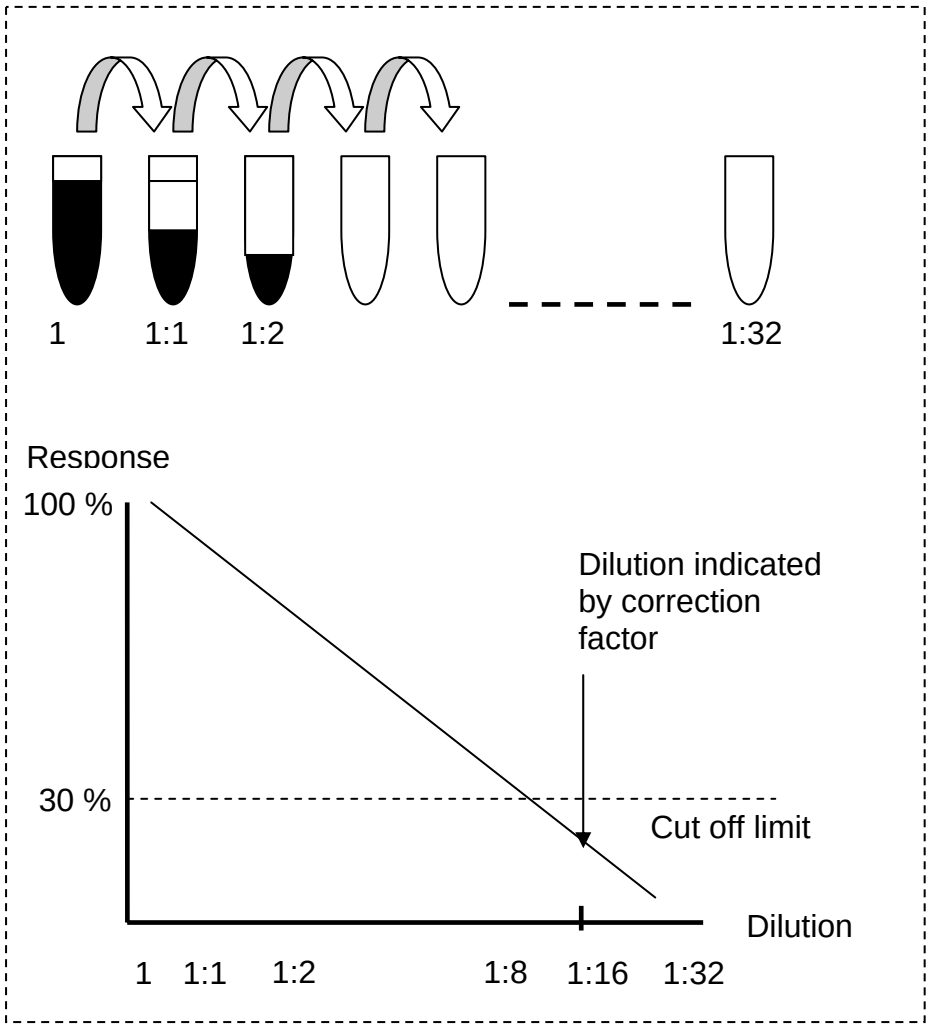

Figure 13. The principle of the dilution series, and the relationship between the dose-response curve and correction factor (CF)

Preparing an extract using the CEN procedures with water or ethanol or Tenax produces a test solution for the biological test systems that is generally much more concentrated than migration levels expected into foods. **Correction factors (CF)** are used therefore to correct for this extra extractive power. CFs for different categories of food-contact applications are derived from a matrix of four parameters that relate the test conditions to the actual use. These are the relative extractive power of the simulant *per se* compared to the food, along with the additional influences of the time, temperature and contact area used in the tests compared to the real food contact application.

5.5.3 Correction Factors (CF)

5.5.3.1. Background to the need for correction factors

It is necessary to relate the extracts prepared, with the migration concentrations expected into foods.

As stated elsewhere in this report, there were 3 guiding principles for preparing the extracts of the paper/board samples for the bioassay procedures:

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

Chemical analysis of the extracts/migrates obtained has revealed that efficient extraction of the NSP series of paper samples occurs. In particular, the tests by total immersion are more severe than applications in contact with foodstuffs. A major task therefore was to make conclusions and recommendations on the applicability of correction factors – as used in EU migration tests of plastics – because the tests used here elicit higher concentrations in the extract than normal or foreseeable conditions of use of P/B in contact with foodstuffs.

Aspects likely to make testing more severe

The tests with solvents or simulants involve total contact whereas in many food contact applications the contact would be only partial, incomplete, or even indirect with an air gap or barrier layer between the food and the paper that will impede or prevent migration.

The tests with solvents or simulants involve more aggressive media which can penetrate the sample exposing the individual fibres and which can have a higher solubility for any substances in the paper/board.

The tests with solvents or simulants are intended to employ a test temperature which is at least the same and is usually higher than the temperature conditions for actual use. The exception to this is high temperature applications such as baking papers. These are discussed later.

Hot or cold water. Immersion in hot or cold water is clearly at least as severe as any food-contact application for migration of ionic and polar substances, except for oven baking where thermal decomposition may take place and give rise to new potential migrants.

Ethanol. Likewise, immersion in 95% ethanol is clearly at least as severe as any food-contact application, except for oven baking.

Tenax. Regarding Tenax as a food simulant, some clear conclusions were drawn in the EU project FAIR-CT98-4318 'Recyclability'. What was clear from all the results that were obtained, is that the migration kinetics from paper/board follow well-known laws. For all the paper/board test materials, for all the model substances, and for all the food and food simulants studied, and at temperatures of 40ºC and above, migration is dominated by partitioning behaviour and diffusion constants are less important. On the other hand, at temperatures around room temperature, diffusion constants play more of a rôle, especially for larger molecules. It can therefore be reasonably expected that at temperatures below room temperature migration is also diffusion controlled. In nearly every case, migration equilibrium was reached rapidly, within 10 days at ambient temperature and within 2 days at higher temperatures. In determining the final (equilibrium) migration levels, the affinity of the different substances for the paper/board relative to the nature of the food, as expressed by the partition coefficient $K_{B/F}$ was found to be crucial. For Tenax the K values ranged between 0.01 and 6 whereas for foodstuffs a range between 3.5 and 85 was applicable. In comparison between Tenax and foodstuffs the partition coefficients between paper or board and Tenax are always approximate one order of magnitude smaller than the partition coefficients between paper or board and real foodstuffs. From these results it was concluded in the 'Recyclability' project that Tenax is a suitable food simulant for testing migration from paper or board samples into foodstuffs - fulfilling the general safety requirement that the migration determined using Tenax is always higher than into foodstuffs.

Aspects likely to make testing less severe

The tests with solvents or simulants use an accelerated test (to avoid undue delay in arriving at the result) and the test time (i.e. the duration) is normally the same or shorter than the conditions of actual use for packaging foods.

Aspects likely to make testing more or less severe?

The tests with solvents or simulants use a fixed ratio of either mass of paper extracted per unit of simulant volume or a fixed area placed in contact with the solvent or simulant. In contrast, paper and board materials are used in a wide variety of applications with a wide range of ratio of the surface area : food mass. Consequently, the fixed ratios used in testing may underestimate or overestimate depending on the exact application intended.

5.5.3.2. How to derive correction factors

In fact, there are very few studies reported in which migration into foods and into simulants has been studied systematically from the same materials. Some studies report composition as well as migration into foods or simulants. Most publications report just composition or simulant results or food migration results.

It must also be recognised that the derivation of correction factors cannot be a precise science, because there will never be a fixed numerical relationship that cover all conditions of time, temperature, exact nature of the food and its corresponding simulant, nature of the substance(s) in- and the general composition of the paper/board sample, and , finally, the ratio of the area : mass. Rather therefore, correction factors have to be derived conventionally from the weight of the evidence available.

Furthermore, It has to be recognised that correction factors cannot be proven for mixtures. For the biological testing, it is the total migrate that is assessed, including unknown substances, and so an element of convention is absolutely necessary in using correction factors and they should still be conservative to allow for this issue of 'unknowns'.

Consider for example the series of Surveys conducted in the UK on migration from paper and board materials into foods [www.food.gov.uk]. The substances range from those freely water-soluble (e.g. acrylamide) to those that are only fat-soluble (e.g. mineral hydrocarbons). The range of foods pack is also wide and, finally, the conditions of use span from freezer temperature to microwave and conventional oven.

Survey of paper and board food contact materials for residual amine monomers from wet strength agents

Curing agents in carton-board food packaging

Grease proofing agents in paper and board

Phthalates in paper and board packaging

Fluorescent whitening agents

Formaldehyde in tea-bag tissue

Mineral hydrocarbons in food contact materials

Paper and board packaging: not likely to be a source of acrylamide in food

Benzophenone from cartonboard

Survey of retail paper and board food packaging materials for polychlorinated biphenyls

Diisopropylnaphthalenes in food packaging made from recycled paper and board

Survey of pentachlorophenol in paper and board packaging used for retail foods

As an example, from the survey of benzophenone migration from cartonboard, the relative migration levels seen under different contact conditions of retail foods was reported [*Food Additives & Contaminants*, 2003, 20, 607-618]. For direct contact and room temperature storage, the average mass fraction migration was 16.1%. The average migration at room temperature storage but with indirect contact was 6-fold lower at 2.7%. The average migration with direct contact but with chilled or frozen storage was again 6-fold lower at 2.6%. Thus, the benefits of a lower storage temperature are the same as the benefits of making only indirect contact. Finally, the average migration with both indirect contact and with chilled or frozen storage was only 0.4% and this is 40-times lower than the room temperature/direct contact average. This shows that to a good approximation, the attenuation effects of indirect contact and of low temperature storage are cumulative; with a 6-fold reduction for indirect contact compared with direct contact, a 6-fold reduction for chilled/frozen storage compared with ambient storage, and a $6 \times 6 \approx 40$-fold reduction for the two contact conditions combined.

However, these factors are for benzophenone only. Different factors would be expected for, say, mineral oils, pentachlorophenol, or inorganic lead.

Surface area correction factor (SACF)

For plastics the conventional SA:M ratio of packaging used for food is 6 dm^2/kg but this is generally recognised as too low and for P/B we will consider the typical ratio to be 10 dm^2/kg. The concentrations on a surface area basis achieved for the *in vitro* tests deviate from this conventional ratio. The water and ethanol tests use a fixed mass of paper extracted and so need to be linked using the grammage (in grams per dm^2, GDMS). The tests with Tenax use a fixed surface area of test sample and so the correction factor is a fixed value.

Hot or cold water extraction procedures employ 40g/L and the extract is used directly in the *in vitro* tests.

- SACF = 4 / GDMS

Ethanol extraction procedure uses 100g/L and then the extract is concentrated to 1 g/mL. Finally, 2% of this is added *in vitro*, giving 20g/L.

- SACF = 2/GDMS

Tenax procedure exposes 1 dm^2/g and the Tenax is then extracted using ethanol and this extract is concentrated to 0.22 dm^2/mL. Finally, 2% of this is added *in vitro* giving 4.4 dm^2/L.

- SACF = 0.5

Food type correction factor (FTCF)

The extraction tests with water and ethanol are by total immersion in the liquid. For foods, the area making intimate contact with the P/B will depend on the physical form of the food and will be lower for powders and solids.

- Liquids FTCF = 1
- Pastes FTCF = 1
- Powders FTCF = 2
- Solids FTCF = 5

The test with Tenax are by single-sided contact. Because migration into Tenax needs only dry contact then no FTRF is appropriate.

- Liquids FTCF = na (Tenax would not be used)

- Pastes FTCF = na (Tenax would not be used)
- Dry powders FTCF = 1
- Dry solids FTCF = 1

Temperature correction factor (TCF)

The extraction tests are at room temperature (ethanol or cold water) or at elevated temperature to suite the application (hot water, high temperature for Tenax). So for chilled foods (short time low temperature) and for frozen foods (much lower temperature but longer storage time) a correction factor is needed

- Chilled or frozen food TCF = 5

Paper and board used in high temperature applications such as baking papers or ovenable board need special consideration. The highest test temperatures used in this work were 50ºC with Tenax and 80ºC with hot water. This does not give the possibility of the formation and subsequent migration of thermal breakdown products from e.g. paper-making chemicals or recycling residues in the paper or board. The test using Tenax could be extended to higher test temperatures and no technical difficult ties are envisaged. This could perhaps be combined with a procedure of pre-heating the test specimen to the temperature of use, followed by extraction with ethanol or water, as appropriate, to allow the global *in vitro* assessment of the non-volatile fraction too.

Contact correction factor (CCF)

If the food does not make direct contact with the paper or board because of the presence of an air gap or some other barrier layer, then the migration into food will be impeded compared to using simulants.

- Direct contact CCF = 1
- Indirect contact (barrier layer or air gap) CCF = 5

Illustration of correction factors are combined

The overall correction factor to be used (CF) is obtained by a combination of the different factors described above. i.e.

CF = SACF x FFCF x TCF x CCF

The result is used to relate the expected migration concentration into foods, with the concentration of all substances ('global assessment') obtained from the extraction procedure(s). i.e.

[Foods] = [Extract] / CF

As an illustration, consider the 5 packaging types in the Table below.

	SUGAR BAG	FILTER PAPER	FAST FOOD BOX	FROZEN FOOD CARTON	FROZEN FOOD CARTON
Nature	Dry➔Tenax	Wet➔Water	Fatty➔EtOH	Moist➔water	Dry➔Tenax
Form	Powder➔1	Liquid➔1	Solid➔5	Solid➔5	Solid➔1
Temperature	Ambient➔1	Hot➔hot➔1	Hot➔1	Frozen➔5	Frozen➔5
Contact	Direct➔1	Direct➔1	Direct➔1	Direct➔1	Direct➔1
SACF	surface area correction factor, calculated case-by-case if specific application is known				
CF=	**1**	**1**	**5**	**25**	**5**

These correction factors are undoubtedly conservative (and 'conventional'). They are based on a consideration of published surveys of DiPNs, PCBs, phthalates, dioxins, FWAs, acrylamide, WSAs, biocides, PCP, metals, etc. Correction factors for extracts prepared for global *in vitro* assessments cannot be derived just arithmetically using specific migration/extraction data but, rather, have to be based on a weight-of-evidence approach. It would be also desirable in the future to extend this concept, to relate not just extraction concentrations with migration concentrations in individual

foods, but with concentrations in the total diet, thereby introducing the true element of consumer exposure considerations.

5.5.5. Interpretation of the results

As pointed above the outcome of the test battery indicates the presence or absence of certain hazards. It should be noted that none of the outcomes leads to automatic rejection of the product, but rather - in case the tests indicate hazards - imply the necessity of further studies in order to really assess the risk.

Genotoxicity

According to the present understanding the most serious of chemical hazards is the genotoxic potential, which is associated with carcinogenicity and for which no actual safety limits can be scientifically established. This is because it is considered that the hazard is not 'thresholded', meaning that there is a theoretical risk of adverse effects even at very low dose. This being the case, CFs are not applicable, because these two tests for genotoxicity are hazard identification tests and are not related to migration levels and dose.

Consequently, in case either Ames test or Comet assay or both give an unequivocal positive response, the alternatives are:

1. to choose to a technologically comparable alternative product, or
2. to do further and more sophisticated genotoxicity assays (mammalian point mutation assays, in vivo and in vitro clastogenicity assays etc).

In case the second alternative is selected, and the further tests are negative, one should make certain that after the application of the levels of agents responsible for suspected genotoxic effects are below detection limit. Consistent positive results in a battery of genotoxicity assays **indicate a genotoxic risk**, and the product should not be used in food contact.

Cytotoxicity

Positive results in cytotoxicity assays indicate that something in the sample damages the cellular functions, and this may or may not have safety implications. However, cytotoxic agents, in contrast to genotoxic ones, are assumed to have a treshold value, below which the effects are not considered

serious. Therefore the CF-concept is applicable here. If the cytotoxic activity disappears at the dilution corresponding the CF identified for the intended application, there is no cause for concern.

If, however, the application of CF does not eliminate the cytotoxicity, then - if selecting another non-cytotoxic product is not a feasible alternative - one should identify the cause of the positive response. **In this case the risk assessment involves carrying the hazard identification further to the actual chemical characterization of the responsible agent(s) - bringing the cause of cytotoxicity from unknown to the realm of known and concentrate the risk assessment on that known chemical.** The final judgement on the suitability of the product for food contact then depends on the safety assessment of the identified chemicals. In case they are well known, the task can be relatively easy and be based on published studies or historical evidence. If the identified compound(s) are less well characterized their safety evaluation may need further toxicological testing, and the decision whether to resort to this or try to find technological alternatives rests on the manufacturer.

The application of the decision tree and CF can be illustrated using some examples of the NSP samples examined in the project.

1. NSP8 (See Table 40)

This sample had only moderate cytotoxic properties (EC_{50} values in the acute cytytoxicity assays or in the RNA synthesis inhibition tests were either not obtained or were near the maximum applicable concentration). The sample was negative both in Ames test and in comet assay giving thus no indication of genotoxicity.

Despite marginal positive effects in cytotoxicity assays the material NSP8 would be judged as suitable for food contact applications with CF=1 (borderline) and certainly for CF=2 and above.

2. NSP5 (see Table 40)

The acute cytotoxicity of this sample was comparable to that of NSP4. However, neither the Ames test nor the Comet assay were positive. Thus the main concern is cytotoxicity. In Figure 14 the dose response curve of this sample extract in Hepa-1 cell assay is given. It can be seen, that sixfold dilution of the maximum tested concentration (2%) to appr. 0.3 % would reduce the cytotoxicity to a level not significantly different from the control. Thus, the correction factor would be 6. This would be sufficient also regarding the RNA-synthesis inhibition (data not shown).

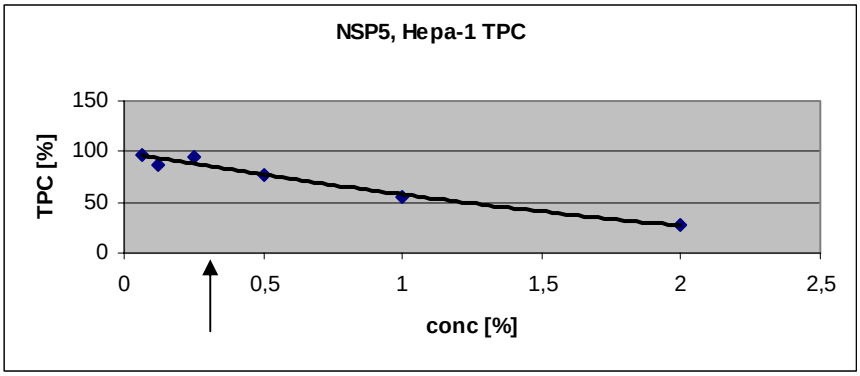

Figure 14. The dose response curve of NSP5 in mouse hepatoma cell (Hepa-1) assay. The concentration (appr. 0.3%) representing the negative response (EC_{20}, see Table 9), obtained by applying CF 6, is marked with an arrow.

In this case the recommendation would be to restrict the use of this paper to applications with CF=6 or above, or to identify the cause of cytotoxicity. This identification was not done during this study. Subsequently it was indicated that sample NSP5 contained a coating material not compatible with ethanol which was used as the solvent. Thus this example illustrates both the need to adjust the extraction conditions according to the material to be studied and - if the cause of cytotoxicty indeed were either the coating material or its breakdown products - to focus the safety assessment on those.

3. NSP4 (see Table 40)

When tested for acute cytotoxicity the sample gave markedly positive results (EC_{50} values were in the range of 0.4 - 0.7 %, depending on the cell line and test system). In the RNA-synthesis inhibition tests with HepG2 and Hela cells the EC_{50} values were in the range of 0.4 - 0.6%). The comet assay was negative at non-cytotoxic concentrations. However, in Ames test the sample proved to be positive with the tester strain TA98 without metabolic activation.

Thus there is an indication of genotoxicity, and further studies to elucidate the actual genotoxic potential of this material would be needed in order to fully assess its suitability for food contact, even after application of CFs.

This sample represents, actually, non-food grade material, and consequently it can be concluded that it was successfully detected in the test battery.

6. DISCUSSION AND CONCLUSION

One of the principal aims of this project was to develop a **battery of tests** that are "quick, cost effective and easy to establish and validate". The tests should also be robust meaning that they can be easily established in different laboratories without affecting the sensitivity or specificity. The tests that were available in the beginning of the project were consistently evaluated against these criteria. Several tests that were considered in the beginning were eventually left out from the final test battery due to:

1 low sensitivity to certain model compounds and lash or difficulties in interpretation of results (bioluminescence test)
2 laboriousness (sister chromatid exhange, non scheduled DNA synthesis)
3 difficulties in standardization between laboratories (micronucleus assay)
4 limited false positives and false negatives, limited information value compared to more established tests (bacterial repair assay)
5 end-points considered too specialized and not relevant for final aims of the project (i.e. immunological effects in lymphocyte tests)

The remaining tests that were used to assess to actual paper and board samples (NSP series) represent both acute cytotoxicity tests (tests with Hep-2 and Hepa-1 cell lines) and tests for sublethal cellular effects such as RNA synthesis inhibition assay (using both HepG2 and HeLa cells) and boar spermatozoan motility inhibition assay. Acute cytotoxicity assays are relatively robust, and can be easily adapted in different laboratories. The RNA-synthesis inhibition test proved to be very sensitive both with model compounds and with actual test extracts. However, its performance requires specially careful and accurate working and experienced laboratory personnel, and cannot be considered as a routine assay for small quality control laboratories. The Boar spermatozoan motility inhibition assay is fast and easy to perform, and very sensitive to some special types of toxins. However, lack of cellular metabolism (protein and nucleic acid synthesis) in sperm cells opens the possibility to false negative responses. Also the interpretation of the test is based on subjective observation, and thus requires some experience.

Preferentially, the acute cytotoxicity assays should be done using the same cell line as in the RNA-synthesis inhibition test. However, all the cell lines used in this project respond rather similarly in cytotoxicity assays, and for screening purposes apparently any cell line that is easy to work with, is applicable. In addition the boar spermatozoan motility inhibition assay was also included as a

complementary test. This test is useful for quick screening purposes, however, particularly negative results obtained in this assay should be confirmed with more traditional cytotoxicity tests. The genotoxic effects were assessed using Ames test (point mutations) and comet assay (physical DNA damage in mammalian cells). This battery of tests was considered as robust, reliable and to represent endpoints relevant to safety assessment.

Tests with model compounds indicate that even these tests, however, often fall short of detecting possible contaminants or known toxic chemicals at the actual concentrations in which they occur in food contact materials or foods. Since the sensitivities of biological systems have limits, these have to be compensated by maximizing the exposure using relevant extraction methods. This is particularly pertinent, when organic solvents such as ethanol are used as a food simulant. The low tolerance of the biological systems towards solvents (generally not more than 2%) necessitates concentration of the extract to make it comparable with water extracts, which can be directly used to reconstitute the cell culture media used in most assays. This was taken into account in this project by modifying the standard ethanol and Tenax extraction procedures (simulating contact with fatty and dry foods, respectively) by both increasing the amount of test material relative to the solvent/absorbent and - in the case of ethanol extracts - by further concentrating the extract.

Of the NSP series only ethanol extracts of samples NSP4, NSP5, NSP8 and NSP13 showed consistent positive results in the different assays, and the effect appeared to be correlated with the chemical content. Extracts with high amounts of chemically variable compounds gave a strong positive response. This effect was clearly seen with extracts of NSP4 and NSP5, the two most toxic samples. The chemicals identified in these extracts represent, in addition to resin acids and other natural components, a variety of chemical contaminants, such as DiPN isomers and phthalates, which were noted for their cytoxicity already when tested as pure compounds (Table 12). Actually, NSP4 represented non-food grade material, and both its cytotoxicity and genotoxicity were clearly demonstrated, when tested as a concentrated ethanol extract. NSP5 has subsequently being reported to contain coating material which is not compatible with ethanol and this material or its break down products (the perfluorinated greaseproofing agents) might be partially responsible for the cytotoxic effects. The fact that the tests applied identified these two most suspicious samples is encouraging considering the eventual use of the tests in safety assessment of food contact materials. In retrospect, inclusion of more non-food grade materials among the NSP samples would have made the outcome of the study even more relevant. The fact that the standard ethanol extract

of NSP4 was non-cytotoxic and non-mutagenic illustrates the need of concentration steps when organic solvents are used as extractants.

Even with these reservations one of the main deliverables of the project, a decision tree based approach for safety assessment using a battery of tests, has been achieved. The combination of cytotoxicity and genotoxicity assays can be represented as a decision tree as shown in the section 5.5.

One other aim of this project was to develop extraction procedures applicable to paper and board with solvent/adsorbent systems simulating different types of foodstuffs and developed from end use applications. The most important outcome was the **standard operating procedure** for preparing extracts intended for toxicity testing. The solvents selected were water for materials intended for wet foods, ethanol as a standard simulant for fatty foods, and Tenax® for dry foods (ethanol is the final solvent for the Tenax® itself). While the standard CEN water extraction procedures designed for chemical migration studies could be applied for materials intended for wet foods, the procedures had to be modified for paper and board intended for fatty or dry foods. The reason for this was simple; while water extracts can be applied in biological tests as undiluted (indeed, the test media can be reconstituted using the extract as the solvent), the tolerance of the biological systems to ethanol is limited to approximately 2 % in solution. Consequently, with both ethanol and Tenax® extracts, the test material/solvent ratio was maximised, and the ethanol extracts were further concentrated. In this way, the concentrations of ethanol dissolved extractables at the applied concentration (max 2 %) in the biological tests were made comparable to those obtained with water extracts (applied at 80 - 90 % concentration).

A further aim of this project was to develop a **risk assessment procedures** based on the scientific evaluation of the results. This was achieved with the design of a decision tree approach for hazard identification based on the selected *in vitro* tests, and the application of the concept of Correction Factors in the interpretation of the results. Correction Factors take into account the necessity of using the most rigorous extraction procedures as possible to reach the detection limits of the biological assays and translate the results into the realistic migration levels that a consumer might meet from actual food packaging. Correction Factors are obtained by taking into account the intended end use, knowledge about the actual migration levels and the nature of the food contact. In practice, they give an indication of any dilution that should be applied to the extract to get an

estimate of the realistic level of migration. The Correction Factor concept was introduced during the project, and obviously needs further refinement in order to be fully applicable.

The external evaluators emphasised the importance of correction factors and that they should be placed on a firm scientific basis. From the few available studies, in which the results of the migrations tests have been compared to concentrations actually found in foods, it can be concluded that the real consumer exposure is much lower (often 1% or less of the amount in the packaging material) than the outcome of extraction trials would indicate, meaning that certain correction factors could be useful in the actual risk assessment. However, more data are obviously needed, before meaningful recommendations on general correction factors applicable to various types of food contact materials can be introduced. This would then create a solid database for the application of correction factors.

7. EXPLOITATION AND DISSEMINATION OF RESULTS INCLUDING POLICY RELATED BENEFITS

7.1. Relevance for Regulation

The project has been necessary in order to launch harmonisation of risk assessment for food contact paper and board at European level. The development of European regulations will harmonise European markets and reduce the risks posed by the introduction of local standards. EU safety compliance, for many food packaging materials, is established by making toxicological assessments of chemical raw materials and then relating the results to human intake by a series of chemical tests and theoretical calculations. Paper and board is manufactured mainly from natural raw materials and, in common with most such products, has a slightly variable composition. Therefore, the objective of the project was to develop a battery of biological tests which assessed the final material rather than the initial, raw materials. By seeking to measure risk under actual food use conditions, it was believed that a new, relevant compliance tool could be added to those already existing within EU legislation. The suggested methodology is in line with the EU Commission's objective of developing a more effective and reliable risk management approach that is less time and resource consuming.

7.2. Standardisation and Validation of Methodology

As part of the dissemination and exploitation of project results to standardization bodies, CEN (the European standardization body), has been contacted and informed about the testing methodology being produced by the project. The project partners agreed to suggest to the Technical Committee CEN/TC 172 "Paper, board and pulps" the following standard "Paper and board intended to come into contact with foodstuffs – Preparation of extracts of paper and board suitable for safety assessment using *in vitro* bioassays".

As part of the validation of the test methods (sperm microassays) an abstract was sent to the First Swift-WFD Workshop on validation of robustness of sensors and bioassays for screening pollutants, Mao, Menorca Spain December 2-3, 2004. The abstract is included in the folder "Communication".

The Comet Assay and RNA Synthesis tests will require official OECD validation. A full request would be made simultaneously by French and Finnish representatives.

7.3. Benefits for industry and consumers

This project represents a pre-normative research effort to address consumer and regulatory concerns by a decision-tree approach using short-term tests with end points relevant to the genuine risk involved in the end-use. The successful outcome enables paper and board producers to promote their products on the basis of sound, transparent, scientific criteria making them highly adapted to to-day's sophisticated, safety conscious food market. Throughout this project it has been possible to establish cooperation between the partners and to create a network between the scientific and industrial communities. Representatives from the paper packaging industry supported the study results and expressed their willingness to cooperate with the regulators and provide the necessary data and background information. They will also continue disseminating the results after the end of the project in order to promote acceptance of the project's concepts among consumer organisations, standards' bodies, regulatory authorities and enforcement institutes.

7.4. Internal and external communication

The project partners placed major emphasis on communication and dissemination. The main effort has been in creating channels for both internal and external communication. Internal communication was important to provide a mutual exchange of ideas and interpretations among and between the representatives of the academic and industrial sector. It served the very important purpose of bringing the groups together and building up networks and trust. The external communication included poster presentations at scientific conferences, presentations in workshops arranged by the packaging industry, creation and regular updating of a web site and production of project leaflets. The main results from the project were presented to an extended audience at the Results Conference on 12.4.2006 in Brussels. All presentations from this conference are included as a separate folder "Biosafepaper Results Conference". Results of the project have been, or will be, peer reviewed and published in scientific journals.

7.5. In the Future

7.5.1. Scientific follow-up

A COST action has been foreseen as the forum to continue the successful co-operation with scientific and industrial partners in the field of safety assessment of paper and board for food contact.

7.5.2. Industrial follow-up

The European paper and board industry is intending to continue its support of the concepts behind the Biosafepaper project. It sees the methodology as an alternative to many of the existing tools used for other food contact materials which is both simpler and more relevant to consumer safety. Paper and board is constructed of mainly naturally occurring materials and it is not possible to manufacture it the same way as many other packaging materials. The Biosafepaper methodology offers a more appropriate and relevant route for establishing its compliance with legislation. The industry is intending to include the Biosafepaper approach in the guidelines it is currently developing for controlling the manufacture of food contact materials and intends to support the transition from pre-normative research to a workable risk management framework.

8. LITERATURE

The references related to the methodology of toxicological testing have been listed at the end of the Section 4. The general background of the field and the relevant references to the pertinent publications are found in connection of the annexed manuscript " The use of short-term biological tests for the safety assessment of paper and board used in food packaging" (see Section 9).

9. ANNEXES

Table W2. Estimated content of identified substances summarized into compound classes and the content of dry matter (DMC) in the CEN water extracts

Compound Class	CEN water extracts from paper and board samples ($\mu g/dm^2$)					
	NSP 6	**NSP 7**	**NSP11**	**NSP18**	**NSP 19**	**NSP 20**
Fatty acids	18.5	15.6	0.0	17.3	8.4	0.1
Dicarboxylic/hydroxy acids	21.4	110.8	4.7	5.7	122.7	5.1
Resin acids	268.1	31.8	4.5	0.0	548.5	46.6
Other compounds identified	36.6	131.7	27.2	56.5	375.6	1.8
Total identified with GCMS	**344.5**	**289.9**	**36.5**	**79.6**	**1055.2**	**53.6**
Dry Matter Content (DMC)	**17,100**	**3,200**	**23,100**	**18,700**	**9,600**	**800**

Table W3. Estimated concentrations of the identified substances in the CEN cold water extract from NSP6

RT (min)	µg/ml	mg/kg P/B	µg/dm2	Best library match
12.08	0.03	0.8	2.1	Dodecanoic acid
13.53	0.02	0.5	1.4	Tetradecanoic acid
15.49	0.05	1.2	3.3	Heptadecanoic acid
15.94	0.08	2.0	5.6	Oleic acid
16.08	0.09	2.1	6.0	Stearic acid
6.58	0.02	0.4	1.2	Acetic acid, 2-hydroxy
7.37	0.04	1.1	2.9	Propanoic acid, 3-hydroxy
9.14	0.09	2.3	6.4	Propanoic acid,2,3-di-hydroxy
10.09	0.04	0.9	2.5	Butanoic acid,3,4-di-hydroxy
10.95	0.08	1.9	5.3	Butanoic acid,2,3,4-tri-hydroxy
13.14	0.04	1.1	3.1	Azelaic acid
16.37	0.06	1.6	4.4	Resin acid
16.51	0.07	1.7	4.7	Resin acid
16.67	0.13	3.2	8.8	Sandaracopimaric acid
16.76	0.21	5.3	14.9	Resin acid
16.88	0.24	6.1	17.0	Isopimaric acid
16.93	0.16	4.1	11.5	Resin acid
17.13	2.17	54.2	151.8	Dehydroabietic acid
17.33	0.78	19.5	54.7	Abietic acid
6.27	0.03	0.7	1.9	2-Methyl-4-keto-pentan-2-ol
12.87	0.13	3.2	8.9	Diethyleneglycol
12.95	0.10	2.6	7.2	iso-Vanillic acid
13.28	0.12	3.1	8.6	4-Hydroxy-3-metoxy-ethanediol
13.76	0.14	3.6	10.1	Alditol
	4.9	**123.0**	**344.5**	**SUM ALL**

Table W4. Estimated concentrations of the identified substances in the CEN cold water extract from NSP7

RT (min)	µg/ml	mg/kg P/B	µg/dm2	Best library match
10.97	0.03	0.6	1.7	Tetradecanoic acid
11.97	0.06	1.6	4.2	Hexadecanoic acid
12.44	0.08	1.9	5.1	Heptadecanoic acid
12.77	0.04	1.0	2.7	Linolic acid
12.89	0.03	0.7	1.8	Octadecanoic acid
13.36	0.08	2.0	5.5	Resin acid
13.43	0.06	1.5	4.1	Resin acid
13.51	0.14	3.4	9.3	Resin acid
13.70	0.15	3.8	10.4	Dehydroabietic acid
14.25	0.04	0.9	2.4	Neoabietic acid
5.53	0.14	3.5	9.7	Propanoic acid, 2-hydroxy
5.71	0.35	8.6	23.5	Acetic acid, 2-hydroxy
6.23	0.20	5.0	13.5	Oxalic acid
7.65	0.14	3.5	9.4	Propanoic acid, 2,3-di-hydroxy
7.90	0.12	3.0	8.2	But-2-endioic acid
8.08	0.07	1.8	4.8	Propandioic acid, 2-hydroxy
8.29	0.05	1.3	3.5	Pentandioic acid, 2-hydroxy
8.36	0.31	7.6	20.8	Butanoic acid, 3,4-di-hydroxy
8.75	0.16	4.1	11.1	Butandioic acid,2-hydroxy
9.61	0.04	0.9	2.5	Butandioic acid 2,3-di-hydroxy
10.68	0.06	1.5	4.0	Azelaic acid
4.69	0.40	10.0	27.3	Boric acid (tri-TMS)
5.46	0.22	5.4	14.7	2-Methyl-4-keto-pentane-2-ol
7.22	0.54	13.6	37.0	Glycerol
7.76	0.21	5.3	14.4	Valeric acid. 5-hydroxy
9.32	0.07	1.7	4.7	Vanillin
9.45	0.08	2.1	5.7	Arabinopyranose (tetra-TMS)
9.82	0.05	1.2	3.4	Arabinofuranose (tetra-TMS)

10.34	0.03	0.7	1.8	H3PO4 (tri-TMS)
10.47	0.12	3.0	8.0	Triethyleneglycol
10.54	0.07	1.8	4.9	Vanillic acid
10.80	0.02	0.6	1.7	3-Vanillylpropanol
14.94	0.05	1.1	3.1	Heneicosane
15.70	0.07	1.9	5.1	Dotriacontane
	4.3	**106.6**	**289.9**	**SUM ALL**

Table W5. Estimated concentrations of the identified substances in the CEN hot water extract fromNSP11

RT (min)	µg/ml	mg/kg P/B	µg/dm2	Best library match
17.13	0.10	2.4	4.8	Dehydroabietic acid
17.06	0.09	2.3	4.5	12-Hydroxy-octadecanoic acid
5.46	0.25	6.3	12.4	Ethyleneglycol
7.95	0.30	7.6	14.9	Pentasiloxane, octadecamethyl
	0.75	**18.7**	**36.6**	**SUM ALL**

Table W6. Estimated concentrations of the identified substances in the CEN cold water extract from NSP18

RT (min)	µg/ml	mg/kg P/B	µg/dm2	Best library match
14.86	0.11	2.8	6.3	Hexadecanoic acid
15.94	0.19	4.8	10.9	Oleic acid
17.06	0.10	2.5	5.7	12-Hydroxy-octadecanoic acid
5.43	0.80	20.1	45.9	Ethyleneglycol
7.95	0.19	4.7	10.6	Pentasiloxane, dodecamethyl
	1.39	**34.8**	**79.4**	**SUM ALL**

Table W7. Estimated concentrations of the identified substances in the CEN cold water extract from NSP19

RT (min)	µg/ml	mg/kg P/B	µg/dm2	Best library match
11.97	0.06	1.5	4.2	Hexadecanoic acid
12.44	0.06	1.4	4.2	Heptadecanoic acid
13.13	0.17	4.3	12.3	Resin acid
13.23	0.12	2.9	8.4	Resin acid
13.36	0.80	20.1	58.3	Resin acid
13.55	1.13	28.2	81.7	Resin acid
13.62	0.26	6.6	19.1	Resin acid
13.70	2.19	54.9	159.1	Dehydroabietic acid
13.86	2.31	57.8	167.6	Abietic acid
14.22	0.58	14.5	41.9	Neoabietic acid
5.54	0.39	9.6	27.9	Propanoic acid, 2-hydroxy
5.71	0.39	9.8	28.6	Acetic acid, 2-hydroxy
6.23	0.41	10.3	30.0	Oxalic acid
7.65	0.15	3.7	10.8	Propanoic acid, 2,3-di-hydroxy
8.36	0.05	1.3	3.8	Butanoic acid, 3,4-di-hydroxy
8.74	0.11	2.8	8.2	Butanedioic acid, 2-hydroxy
9.60	0.09	2.4	6.9	Butanedioic acid, 2,3-di-hydroxy
10.67	0.09	2.3	6.5	Azelaic acid
4.72	2.71	67.8	196.6	Boric acid (tri-TMS)
5.46	0.52	12.9	37.4	2-Methyl-4-keto-pentane-2-ol
7.22	0.72	18.0	52.2	Glycerol
9.32	0.11	2.8	8.1	Vanillin
9.44	0.09	2.1	6.2	Arabinopyranose, (tetra-TMS)
10.53	0.07	1.8	5.2	Isovanillinic acid
15.11	0.74	18.6	54.0	Triethyleneglycol
17.63	0.16	4.0	11.7	Tetraethyleneglycol
19.82	0.06	1.4	4.1	Pentaethyleneglycol
	14.6	**363.9**	**1055.2**	**SUM ALL**

Table W8. Estimated concentrations of the identified substances in the CEN cold water extract from NSP20

RT (min)	µg/ml	mg/kg P/B	µg/dm2	Best library match
16.08	0.02	0.5	0.1	Stearic acid
6.61	0.12	3.0	0.8	Acetic acid. 2-hydroxy
7.37	0.10	2.5	0.7	Propanoic acid. 3-hydroxy
9.15	0.05	1.2	0.3	Propanoic acid.2.3-di-hydroxy
10.08	0.35	8.8	2.5	Butanoic acid.3.4-di-hydroxy
10.59	0.04	0.9	0.3	Butanedioic acid
10.87	0.02	0.4	0.1	Hexanedioic acid
10.94	0.05	1.3	0.4	Butanoic acid.2.3.4-tri-hydroxy
16.37	0.32	8.1	2.3	Resin acid
16.51	0.25	6.3	1.8	Resin acid
16.67	0.12	2.9	0.8	Sandaracopimaric acid
16.76	0.12	3.0	0.8	Resin acid
16.93	0.66	16.5	4.6	Isopimaric acid
17.02	0.39	9.9	2.8	Resin acid
17.13	2.79	69.8	19.5	Dehydroabietic acid
17.21	0.10	2.6	0.7	Resin acid
17.33	1.74	43.4	12.2	Abietic acid
17.81	0.15	3.9	1.1	Neoabietic acid
5.31	0.15	3.6	1.0	Boric acid (tri-TMS)
6.27	0.08	2.1	0.6	2-Methyl-4-keto-pentan-2-ol
11.34	0.02	0.4	0.1	Vanillin
12.95	0.02	0.4	0.1	iso-Vanillic acid
	7.7	**191.6**	**53.6**	**SUM ALL**

Table 17. Estimated concentrations of the substances in the concentrated ethanol extract of NSP4

RT (min)	µg/ml (= mg/kg P/B)	µg/dm^2	Best library match
6.06	0.6	1.7	hexanal
9.29	0.5	1.4	2-ethyl-1-hexanol
10.49	1.7	5	nonanal
10.54	0.6	1.8	no good library match
14.72	2.4	7	vanillin
16.00	2.0	6	pentadecane
16.09	1.9	6	no good library match
16.25	5.6	17	2-phenylphenol
17.11	3.1	9	diethyl phthalate
17.21	1.9	6	hexadecane
17.68	2.4	7	benzophenone
18.16	68	210	DIPN isomers
18.40	5.6	17	heptadecane
18.70	150	450	DIPN isomers
19.02	27	81	tetramethyl biphenyl isomer
19.49	9.7	29	octadecane
20.13	120	360	diisobutyl phthalate
20.54	4.1	12	nonadecane
21.10	37	110	dibutyl phthalate
21.56	6.9	21	eicosane
22.48	16	49	octadecenoic acid, methyl ester
22.81	21	62	9-octadecenoic acid
23.02	6.7	20	octadecanoic acid
23.14	4.6	14	2-(phenylmethoxy)naphthalene
23.18	16	49	bisphenol A
23.60	22	66	bis(2-ethylhexyl) fumarate
23.79	2.6	8	4-benzyl biphenyl
24.27	18	54	tricosane
24.66	16	49	methyl dehydroabietate
25.10	16	49	tetracosane
25.48	38	110	dehydroabietic acid
25.74	5.1	15	2-(methoxymethyl)-2-phenyl-1,3-dioxolane
25.91	41	120	pentacosane
26.05	2.4	7.2	dicyclohexyl phthalate
26.15	29	89	bis(2-ethylhexyl) phthalate
26.58	2.9	8.8	7-oxodehydroabietic acid, methyl ester

26.69	17	52	hexacosane
27.45	18	53	heptacosane
28.33	8	24	octacosane
29.36	5.5	17	nonacosane
	757	**2274**	**SUM ALL**

Table 18. Estimated concentrations of the substances in the concentrated ethanol extract of NSP5

RT (min)	µg/ml (= mg/kg P/B)	µg/dm^2	Best library match
6.17	0.9	4.9	hexanal
6.20	0.6	3.3	perfluoro compound
7.76	2.0	11	perfluoro compound
8.25	0.2	1.1	no good library match
9.22	1.5	8.6	perfluoro compound
9.35	1.2	6.8	2-ethyl-1-hexanol
9.44	290	1700	1-methyl-2-pyrolidinone
9.95	1.1	6.0	acetophenone
12.05	1	5.6	perfluoro compound
12.17	0.3	1.8	no good library match
12.28	0.3	1.4	no good library match
12.40	0.6	3.4	no good library match
12.60	0.4	2.1	no good library match
13.58	1.2	6.9	perfluoro compound
14.72	3.9	22	vanillin
16.00	1.7	9.5	pentadecane
16.08	1.1	6.5	no good library match
16.26	9.3	53	2-phenylphenol
16.72	2.1	12	dodecanoic acid
17.08	3.4	19	diethyl phthalate
17.22	4.1	23	hexadecane
17.65	2.6	15	benzophenone
18.16	12	69	DIPN isomers
18.40	12	70	heptadecane
18.70	25	140	DIPN isomers
19.49	16	83	octadecane
20.13	65	370	diisobutyl phthalate
20.53	4.4	25	nonadecane
21.11	10	58	dibutyl phthalate
21.53	1.1	6.5	eicosane
21.71	31	180	1-cyclohexene-1-carboxylic acid, 4-(1,5-dimethyl-3-oxohexyl)-, methyl ester [S-(R*,R*)]-
22.17	4.6	26	1-naphthalenepropanol, alpha.-ethenyldecahydro-.alpha.,5,5,8a-tetramethyl-2-methylene-, [1S-

			(1.alpha.(S*),4a.beta.,8a.alpha.)]-
22.34	48	280	no good library match
22.48	4.1	24	octadecenoic acid, methyl ester
22.70	2.6	15	no good library match
22.76	5.2	30	9,12-octadecedienoic acid
22.82	28	160	9-octadecenoic acid
22.98	1.4	8.0	no good library match
23.03	3.9	22	octadecanoic acid
23.39	13	74	docosane
23.60	11	63	bis(2-ethylhexyl)fumarate
24.17	49	280	eicosene
24.26	8.2	47	tricosane
24.37	5.1	29	no good library match
24.67	13	77	methyl dehydroabietate
25.10	10	58	tetracosane
25.48	21	120	dehydroabietic acid
25.87	82	470	no good library match
26.15	20	120	bis(2-ethylhexyl) phthalate

Table 19. Estimated concentrations of the substances in the concentrated ethanol extract of NSP8

RT (min)	µg/ml (= mg/kg P/B)	µg/dm^2	Best library match
6.26	0.2	0.33	hexanal
20.13	2.2	3.5	diisobutyl phthalate
22.34	5.1	8.2	octadecene
23.26	0.8	1.3	no good library match
23.75	2.8	4.5	4-(2-(4-nitrophenyl)ethylbenzamine)
24.04	1.1	1.8	no good library match
24.17	7.8	13	eicosene
24.66	1.1	1.8	methyl dehydroabietate
25.85	11	18	no good library match
26.15	0.7	1.0	bis(2-ethylhexyl) phthalate
26.24	1.3	2.0	2,2'-bis(p-methoxyphenyl)-1,1-dichloroethylene
26.39	6.4	10	gamma-ergostanol
28.47	29	47	stigmast-7-en-3-ol, (3.beta.,5.alpha.,24S)-
28.76	7.7	12	no good library match
30.79	3.8	6.1	no good library match
	81	**131**	**SUM ALL**

Table 20. Estimated concentrations of the substances in the concentrated ethanol extract of NSP13

RT (min)	µg/ml (= mg/kg P/B)	µg/dm^2	Best library match
6.09	0.5	4.1	hexanal
7.19	0.4	3.3	no good library match
14.69	12	93	vanillin
15.79	1.1	9	1-(3-hydroxy-4-methoxyphenyl)ethanone
17.20	5.6	42	2,6-dimethoxy-4-(2-propenyl)phenol
17.89	3.6	27	4-hydroxy-3,5-dimethoxybenzaldehyde
18.13	1.5	11	DIPN isomers
18.39	0.8	6	no good library match
18.71	0.5	3.4	DIPN isomers
20.13	13	99	diisobutyl phthalate
21.11	8.5	64	no good library match
21.82	3.8	29	1H-naphtho(2,1-b)pyran, 3-ethenyldodecahydro-3,4a,7,7,10a-pentamethyl-,[3R-(3.alpha.,4a.beta.,6a.alpha., 10a.beta., 10b.alpha.)]
22.34	28	210	octadecene
22.76	4.3	33	9,12-octadecedienoic acid
22.81	17	130	9-octadecenoic acid
23.19	0.5	4.1	bisphenol A
23.26	1.7	13	pregn-14-ene, (5.beta.)-
23.60	2.4	18	bis(2-ethylhexyl) fumarate
23.75	2.6	20	4-(2-(4-nitrophenyl)ethylbenzamine)
24.04	3.5	26	no good library match
24.17	13	99	eicosene
24.26	1.6	12	tricosane
24.43	1.3	10	no good library match
24.66	2.9	22	methyl dehydroabietate
25.10	3.0	23	tetracosane
25.47	2.8	21	dehydroabietic acid
25.86	49	370	no good library match
26.15	5.8	44	bis(2-ethylhexyl) phthalate
26.68	4.7	36	hexacosane
27.11	3.8	29	no good library match
27.45	5.6	43	heptacosane
28.32	2.0	15	octacosane
28.46	15	120	stigmast-7-en-3-ol, (3.beta.,5.alpha.,24S)-

28.82	2.4	18	no good library match
	224	**1707**	**SUM ALL**

Table 21. Estimated concentrations of the substances in the EN1186-15 ethanol extract of NSP4

RT (mins)	µg/ml	µg/dm^2	Best library match
15.51	0.35	17	Tetradecane
16.83	0.26	13	Pentadecane
17.63	0.37	19	o-Hydroxybiphenyl
18.06	0.21	10	Hexadecane
18.35	0.39	19	Isopropyl dodecanoate
18.78	0.28	14	Benzophenone
19.23	5.4	270	DIPN isomers and heptadecane
19.79	10	500	DIPN isomers
20.10	1.8	92	2,2',5,5'-Tetramethyl-1,1'-biphenyl
20.35	1.1	56	Benzyl benzoate and octadecane
21.11	7.1	360	Diisobutyl phthalate
22.09	1.7	83	Dibutyl phthalate
23.40	1.2	59	9-Octadecenoic acid, methyl ester
24.59	0.76	38	Bis-(2-ethylhexyl) fumarate
25.22	1.0	51	Tricosane
25.82	0.97	48	Methyl dehydroabietate
26.07	1.6	80	Tetracosane
26.90	1.9	94	Pentacosane
27.20	1.9	96	Bis-(2-ethylhexyl) phthalate
27.73	1.7	83	Hexacosane
28.70	1.6	81	Heptacosane

Table 22. Estimated concentrations of the substances in the EN1186-15 ethanol extract of NSP13

RT (mins)	µg/ml	µg/dm^2	Best library match
16.00	3.4	170	Vanillin
18.16	0.36	18	No good match
18.33	0.58	29	2,6-Dimethoxy-4-(2-propenyl)-phenol
19.22	1.2	58	4-Hydroxy-3,5-dimethoxybenzaldehyde
21.11	2.1	110	Diisobutyl phthalate
23.85	4.8	240	Octadecene
25.79	1.9	93	Unspecified alkene
27.20	0.40	20	Bis-(2-ethylhexyl) phthalate
27.54	2.1	100	Unspecified alkene
27.72	0.47	23	No good match

Table 23. Estimated concentrations of the substances in the low molecular weight fraction of the ethanol extract of NSP4

RT (min)	µg/ml	µg/dm^2	Best library match
13.77	78	230	siloxane
15.97	49	150	siloxane
17.94	17	53	siloxane
18.60	61	180	diisopropylnaphthalene isomers
19.15	140	420	diisopropylnaphthalene isomers
19.46	27	82	tetramethyl biphenyl isomer
19.83	13	40	ethyl tetradecanoate
20.55	100	310	diisobutyl phthalate *
21.52	21	64	dibutyl phthalate *
21.87	71	220	ethyl hexadecanoate *
23.50	100	300	ethyl octadecenoate *
23.58	38	120	bisphenol A *
25.09	8.4	30	dehydroabietic acid, methyl ester
26.56	41	120	di-(2-ethylhexyl) phthalate *
	764	**2319**	**SUM ALL**

* low levels detected in the blank too

Table 24. Estimated concentrations of the substances in Tenax exposed to NSP1

RT (min)	µg/ml	µg/dm^2	Best library match
5.82	0.055	0.24	1-pentanol
5.86	0.033	0.15	butanoic acid
6.16	0.16	0.71	hexanal
7.44	0.029	0.13	heptanal
7.47	0.046	0.20	2-butoxyethanol
8.36	0.20	0.89	benzaldehyde
8.40	0.037	0.17	no good library match
8.69	0.035	0.15	1,2-diethylbenzene
8.94	0.036	0.16	octanal
8.99	0.029	0.13	2,2,4,6,6-pentamethyl-3-heptene
9.30	0.12	0.53	2-ethyl-1-hexanol
9.93	0.039	0.17	acetophenone
13.35	0.24	1.1	tridecane
14.71	0.48	2.1	tetradecane
14.99	0.14	0.62	1,4-methanoazulene, decahydro-4,8,8-trimethyl-9-methylene-, [1S-(1.alpha.,3a.beta.,4.alpha.,8a.beta.)]-
15.99	0.58	2.6	pentadecane
17.22	1.0	4.6	hexadecane
17.49	0.23	1.0	1-methylethyl dodecanoate
17.75	0.56	2.5	branched alkane
18.15	2.4	11	DIPN isomers
18.40	2.3	10	heptadecane
18.71	4.7	21	DIPN isomers
19.49	2.0	9.1	octadecane
20.54	0.61	2.7	nonadecane
21.53	0.34	1.5	eicosane
22.48	0.39	1.7	heneicosane
23.39	0.39	1.7	docosane
24.27	0.30	1.3	tricosane
25.10	0.23	1.0	tetracosane
25.90	0.065	0.29	pentacosane
26.69	0.13	0.59	hexacosane
27.46	0.071	0.31	heptacosane
	18	**80**	**SUM ALL**

Table 25. Estimated concentrations of the substances in Tenax exposed to NSP2

RT (min)	µg/ml	µg/dm^2	Best library match
5.58	0.052	0.23	triethyl borate
5.80	0.037	0.16	1-pentanol
6.15	0.23	1.0	hexanal
6.28	0.021	0.09	no good library match
7.26	0.027	0.12	no good library match
7.43	0.054	0.24	heptanal
8.36	0.45	2.0	benzaldehyde
8.94	0.10	0.46	octanal
9.93	0.27	1.2	acetophenone
13.36	0.18	0.81	tridecane
14.72	0.99	4.4	tetradecane
16.00	1.5	6.8	pentadecane
16.10	0.67	3.0	tributyl phosphate
17.08	0.86	3.8	diethyl phthalate
17.21	2.2	9.9	hexadecane
17.75	1.3	5.9	branched alkane
18.16	10	46	DIPN isomers
18.40	5.7	25	heptadecane
18.71	20	91	DIPN isomers
19.49	6.5	29	octadecane
20.14	25	109	diisobutyl phthalate
20.54	2.0	9.1	nonadecane
21.10	6.9	31	dibutyl phthalate
21.25	0.86	3.8	no good library match
21.53	1.3	6.0	no good library match
21.64	0.68	3.0	1H-naphtho(2,1-b)pyran, 3-ethenyldodecahydro-3,4a,7,7,10a-pentamethyl-, [3S-(3.alpha.,4a.alpha,6a.beta.,10a.alpha.,10b.beta.)]
21.85	1.3	5.6	1H-naphtho(2,1-b)pyran, 3-ethenyldodecahydro-3,4a,7,7,10a-pentamethyl-,[3R-(3.alpha.,4a.beta.,6a.alpha., 10a.beta., 10b.alpha.)]
22.17	1.3	5.7	1-naphthalenepropanol, .alpha.-ethenyldecahydro-.alpha.,5,5,8a-tetramethyl-2-methylene-, [1S-(1.alpha.(S*),4a.beta.,8a.alpha.)]
22.48	7.2	32	octadecenoic acid, methyl ester
22.70	1.1	4.8	no good library match
22.75	0.58	2.6	no good library match

23.15	1.2	5.5	no good library match
23.39	2.7	12	docosane
24.27	3.1	14	tricosane
24.66	2.8	12	methyl dehydroabietate
24.77	0.60	2.6	no good library match
25.00	3.7	17	bis (2-ethylhexyl) hexadecanoate
25.10	3.3	15	tetracosane
25.91	5.0	22	pentacosane
26.05	2.3	10	dicyclohexyl phthalate
26.15	3.0	13	bis (2-ethylhexyl) phthalate
26.59	0.65	2.9	7-oxodehydroabietic acid, methyl ester
26.69	3.3	15	hexacosane
27.45	2.5	11	heptacosane
28.33	1.5	6.5	octacosane
29.36	1.2	5.3	nonacosane
30.60	0.68	3.0	triacontane
	137	**611**	**SUM ALL**

Table 26. Estimated concentrations of the substances in Tenax exposed to NSP3

RT (min)	µg/ml	µg/dm^2	Best library match
8.28	0.26	1.2	benzaldehyde
8.36	0.088	0.39	No good library match
8.89	0.049	0.22	octanal
9.43	0.24	1.1	1-methyl-2-pyrrolidinone
9.89	0.075	0.33	acetophenone
14.70	0.55	2.5	tetradecane
15.99	1.3	6.0	pentadecane
17.12	0.87	3.9	pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl, isobutyl ester
17.25	2.4	11	hexadecane
17.53	0.97	4.3	1-methylethyl-dodecanoate
17.75	1.5	6.6	branched alkane
18.10	4.1	18	DIPN isomers
18.40	5.6	25	heptadecane
18.70	6.4	28	DIPN isomers
19.50	5.8	26	octadecane
20.13	6.4	28	diisobutyl phthalate
20.54	2.1	9.2	nonadecane
21.10	0.71	3.2	dibutyl phthalate
22.48	4.0	18	octadecenoic acid, methyl ester
23.15	0.92	4.1	no good library match
23.39	2.6	12	no good library match
24.27	2.1	9.4	tricosane
24.67	2.2	9.4	methyl dehydroabietate
25.10	3.0	13	tetracosane
25.91	3.6	16	pentacosane
26.15	5.2	23	bis(2-ethylhxyl) phthalate
26.59	0.29	1.3	7-oxodehydroabietic acid, methyl ester
26.69	2.3	10	hexacosane
27.46	1.8	7.9	heptacosane
28.33	0.96	4.3	octacosane
29.36	0.64	2.9	nonacosane
30.61	0.37	1.7	triacontane
	69	**308**	**SUM ALL**

Table 27. Estimated concentrations of the substances in Tenax exposed to NSP9

RT (min)	µg/ml)	µg/dm^2)	Best library match
6.44	0.098	0.43	hexanal
7.41	0.027	0.12	heptanal
8.93	0.057	0.26	octanal
9.30	0.081	0.36	2-ethyl-1-hexanol
9.78	0.034	0.15	no good library match
9.93	0.044	0.20	acetophenone
12.02	0.053	0.23	decanal
12.29	0.030	0.13	no good library match
12.39	0.053	0.23	benzothiazole
12.60	0.068	0.30	1,1,3,5-tetramethylcyclohexane
12.84	0.047	0.21	no good library match
14.72	0.063	0.28	tetradecane
14.99	0.19	0.82	1,4-methanoazulene, decahydro-4,8,8-trimethyl-9-methylene-, [1S-(1.alpha.,3a.beta.,4.alpha.,8a.beta.)]-
16.00	0.062	0.27	pentadecane
17.09	0.15	0.67	diethyl phthalate
17.22	0.14	0.62	hexadecane
18.16	1.1	4.9	DIPN isomers
18.39	0.49	2.2	heptadecane
18.70	2.6	12	DIPN isomers
19.02	0.28	1.2	tetramethyl biphenyl isomer
19.49	0.70	3.1	octadecane
19.69	0.14	0.60	no good library match
20.13	17	73	diisobutyl phthalate
20.54	0.37	1.7	nonadecane
20.74	0.17	0.75	no good library match
21.09	2.2	9.7	dibutyl phthalate
21.46	0.21	0.93	ethyl hexadecanoate
21.53	0.27	1.2	eicosane
22.48	1.1	5.0	heneicosane
23.39	1.8	8.0	docosane
24.27	2.9	13	tricosane
24.67	1.3	5.7	methyl dehydroabietate
25.10	4.3	19	tetracosane
25.91	4.9	22	pentacosane
26.15	1.5	6.5	bis(2-ethylhexyl) phthalate

26.69	4.4	19	hexacosane
27.46	3.4	15	heptacosane
28.33	1.8	7.9	octacosane
28.46	0.47	2.1	2,6,10,14,18,22-tetracosahexaene, 2,6,10,15,19,23-hexamethyl-, (all-E)-
29.37	1.2	5.5	nonacosane
30.60	0.51	2.3	triacontane
	56	**248**	**SUM ALL**

Table 28. Estimated concentrations of the substances in Tenax exposed to NSP10

RT (min)	µg/ml	µg/dm^2	Best library match
14.71	0.16	0.72	tetradecane
15.99	0.20	0.87	pentadecane
17.21	0.34	1.5	hexadecane
18.20	9.1	40	DIPN isomers
18.40	0.96	4.3	heptadecane
18.71	19	83	DIPN isomers
18.99	3.5	16	tetramethyl biphenyl isomer
19.50	1.3	5.6	octadecane
20.13	14	61	diisobutyl phthalate
20.54	0.75	3.4	nonadecane
21.09	2.3	10	dibutyl phthalate
21.53	0.93	4.1	eicosane
22.48	1.2	5.5	heneicosane
23.14	0.66	2.9	2-(phenylmethoxy)naphthalene
23.40	2.1	9.3	docosane
24.27	2.4	11	tricosane
24.66	0.51	2.3	methyl dehydroabietate
25.10	2.4	11	tetracosane
25.91	1.9	8.5	pentacosane
26.69	1.1	4.9	hexacosane
27.46	0.65	2.9	heptacosane
28.33	0.27	1.2	octacosane
29.36	0.11	0.48	nonacosane
	66	**290**	**SUM ALL**

Table 29. Estimated concentrations of the substances in Tenax exposed to NSP12

RT (min)	µg/ml	µg/dm^2	Best library match
5.96	0.0049	0.022	hexanal
7.35	0.029	0.13	heptanal
8.30	0.098	0.44	benzaldehyde
8.90	0.041	0.18	octanal
9.91	0.050	0.22	acetophenone
13.35	0.16	0.72	tridecane
14.71	0.65	2.9	tetradecane
14.99	0.15	0.67	1,4-methanoazulene, decahydro-4,8,8-trimethyl-9-methylene-, [1S-(1.alpha.,3a.beta.,4.alpha.,8a.beta.)]-
15.99	0.74	3.3	pentacosane
17.08	0.18	0.81	diethyl phthalate
17.21	0.35	1.6	hexadecane
17.49	0.30	1.3	1-methylethyl dodecanoate
18.19	1.6	6.9	DIPN isomers
18.40	0.54	2.4	heptadecane
18.71	3.5	15	DIPN isomers
19.03	0.25	1.1	tetramethyl biphenyl isomer
19.49	0.76	3.4	octadecane
20.13	11	50	diisobutyl phthalate
20.53	0.48	2.1	nonadecane
21.10	4.7	21	dibutyl phthalate
21.53	0.78	3.5	eicosane
22.48	2.1	9.2	heneicosane
23.39	2.6	12	tricosane
24.27	3.2	14	tetracosane
24.66	0.74	3.3	methyl dehydroabietate
25.10	5.1	23	tetracosane
25.91	5.7	25	pentacosane
26.15	15	66	bis(2-ethylhexyl) phthalate
26.69	4.5	20	hexacosane
27.46	2.9	13	heptacosane
28.33	1.6	7.2	octacosane
29.36	1.2	5.3	nonacosane
30.61	0.68	3.0	triacontane
	71	**319**	**SUM ALL**

Table 30. Estimated concentrations of the substances in Tenax exposed to NSP14

RT (min)	µg/ml	µg/dm^2	Best library match
5.48	0.33	1.5	triethyl borate
5.72	0.059	0.26	no good library match
6.11	0.082	0.36	1-pentanol
6.23	0.093	0.41	2,3-butanediol
6.39	0.27	1.2	hexanal
7.55	0.038	0.17	heptanal
8.96	0.082	0.37	octanal
9.30	0.14	0.61	2-ethyl-1-hexanol
9.78	0.050	0.22	no good library match
9.92	0.089	0.40	acetophenone
13.33	0.28	1.3	tridecane
14.69	0.88	3.9	tetradecane
15.97	1.4	6.2	pentadecane
17.20	1.3	5.6	hexadecane
17.47	2.0	9.1	1-methylethyl dodecanoate
18.12	8.4	38	DIPN isomers
18.37	2.8	13	heptadecane
18.64	15	68	DIPN isomers
19.50	3.3	15	octadecane
20.06	22	98	diisobutyl phthalate
20.54	0.90	4.0	nonadecane
21.10	1.8	8.0	dibutyl phthalate
21.53	0.71	3.2	eicosane
22.48	2.3	10	heneicosane
23.39	1.1	5.1	docosane
24.26	1.1	4.8	tricosane
24.66	0.18	0.80	methyl dehydroabietate
25.10	0.87	3.9	tetracosane
25.91	0.35	1.6	pentacosane
26.69	0.11	0.49	hexacosane
	68	**305**	**SUM ALL**

Table 31 Estimated concentrations of the substances in Tenax exposed to NSP15

RT (min)	µg/ml	µg/dm^2	Best library match
21.25	0.30	1.3	no good library match
21.45	0.25	1.1	ethyl hexadecanoate
24.66	0.18	0.82	methyl dehydroabietate
26.15	9.0	40	bis(2-ethylhexyl) phthalate
	10	**43**	**SUM ALL**

Table 32. Estimated concentrations of the substances in Tenax exposed to NSP16

RT (min)	µg/ml	µg/dm^2	Best library match
17.53	0.85	3.8	1-methylethyl dodecanoate
18.39	0.59	2.6	heptadecane
19.50	0.69	3.1	octadecane
20.54	0.16	0.71	nonadecane
20.79	1.97	8.8	methyl hexadecanoate
21.53	0.032	0.14	eicosane
21.77	0.020	0.087	methyl heptadecanoate
22.02	0.046	0.21	no good library match
22.08	0.030	0.13	no good library match
22.48	9.3	41	octadecenoic acid, methyl ester isomers
22.72	1.4	6.0	methyl octadecanoate
	15	**67**	**SUM ALL**

Table 33. Estimated concentrations of the substances in Tenax exposed to NSP17

RT (min)	µg/ml	µg/dm^2	Best library match
5.80	0.21	0.94	1-pentanol
6.15	2.7	12	hexanal
6.97	0.046	0.20	no good library match
7.01	0.052	0.23	pentanoic acid
7.11	0.069	0.31	2-methylhexanal
7.25	0.062	0.28	2-heptanone
7.43	0.12	0.53	heptanal
8.23	0.18	0.80	2-heptenal
8.36	0.70	3.1	benzaldehyde
8.94	0.19	0.83	octanal
9.47	0.13	0.57	3-octen-2-one
9.59	0.11	0.49	no good library match
9.79	0.73	3.3	no good library match
9.93	0.28	1.2	acetophenone
12.59	0.60	2.7	1,1,3,5-tetramethylcyclohexane
12.83	0.16	0.69	no good library match
13.92	0.086	0.38	no good library match
14.07	0.082	0.37	no good library match
14.25	0.11	0.47	no good library match
17.63	0.17	0.75	alpha-cubebene
17.86	0.13	0.60	4-(2,6,6-trimethyl-cyclohex-1-enyl)-butan-2-ol
18.29	0.22	0.96	no good library match
19.68	8.7	39	3-octadecene
20.13	0.28	1.2	diisobutyl phthalate
21.23	0.95	4.2	no good library match
21.45	0.37	1.7	ethyl hexadecanoate
21.64	1.6	7.0	1H-naphtho(2,1-b)pyran, 3-ethenyldodecahydro-3,4a,7,7,10a-pentamethyl-,(3S-(3.alpha.,4a.alpha,6a.beta, 10a.alpha, 10b.beta)
21.85	2.7	12	1H-naphtho(2,1-b)pyran, 3-ethenyldodecahydro-3,4a,7,7,10a-pentamethyl-,(3S-(3.alpha.,4a.alpha,6a.beta, 10a.alpha, 10b.beta)
22.08	1.5	6.7	thunbergol
22.16	0.70	3.1	7-isopropyl-1,1,4a-trimethyl-1,2,3,4,4a,9,10,10a-octahydrophenanthrene
22.39	1.2	5.2	phenanthrene, 1,2,3,4,4a,9,10,10a-octahydro-1,1,4a-trimethyl-7-(1-methylethyl)-, (4aS-trans)
22.69	4.1	18	no good library match

22.98	1.2	5.2	no good library match
23.10	0.47	2.1	no good library match
23.37	2.5	11	no good library match
23.63	0.32	1.4	phenanthrene, 1,2,3,4,4a,9,10,10a-octahydro-1,1,4a-trimethyl-7-(1-methylethyl)-, (4aS-trans) – same match as 22.39 minutes, possible isomers
23.82	0.23	1.0	3-buten-1-one, 4-[2,6,6-trimethyl-1(or 2)-cyclohexen-1-yl]-
24.37	0.41	1.8	1-phenanthrenecarboxylic acid, 7-ethenyl-1,2,3,4,4a,4b,5,6,7,8,10,10a-dodecahydro-1,4a,7-trimethyl-, methyl ester [1R-(1.alpha.,4a.beta.,4b.alpha.,7.alpha.,10a.alpha.)]
24.66	3.9	18	methyl dehydroabietate
24.93	0.16	0.69	1-phenanthrenemethanol,1,2,3,4,4a,9,10,10a-octahydro-1,4a-dimethyl-7-(1-methylethyl)-, [1S-(1.alpha.,4a.alpha.,10a.beta.)]
25.93	0.81	3.6	no good library match
26.15	0.16	0.72	bis(2-ethylhexyl) phthalate
26.20	0.25	1.1	15-hydroxydehydroabietic acid, methyl ester
26.58	0.48	2.1	7-oxodehydroabietic acid, methyl ester
26.68	0.11	0.50	no good library match
27.17	0.13	0.58	no good library match
27.45	0.29	1.3	no good library match
28.46	0.23	1.0	2,6,10,14,18,22-tetracosahexaene, 2,6,10,15,19,23-hexamethyl-, (all-E)-
	41	**182**	**SUM ALL**

Table 34. Concentration of semi-volatile substances in Tenax exposed to NSP4

RT (min)	µg/ml	µg/dm^2	Best library match
10.89	0.29	1.3	Acetophenone
15.17	0.30	1.4	Branched alkane
15.52	1.1	5.0	Tetradecane
15.91	0.18	0.80	Longicyclene
16.83	1.4	6.3	Pentadecane
16.95	0.57	2.5	Tributyl phosphate
17.44	0.22	0.97	Branched alkane
17.60	0.33	1.5	Branched alkane and 1-phenoxy-2-(2-propenyl)-benzene
17.92	0.98	4.4	Propanoic acid, 2-,1-methyl-1-(1,1-dimethylethyl)-2-methyl-1,3-propanediyl ester
18.06	1.7	7.6	Hexadecane
18.36	1.7	7.5	Isopropyl dodecanoate
18.59	0.65	2.9	Branched alkane
19.23	18	81	DIPN isomers and heptadecane
19.80	26	120	DIPN isomers
20.11	4.0	18	2,2'5,5'-Tetramethyl-1,1'-biphenyl
20.35	3.4	15	Octadecane
20.81	0.32	1.4	Branched alkane
21.11	17	77	Diisobutyl phthalate
21.41	1.4	6.2	Nonadecane
22.10	1.5	6.8	Dibutyl phthalate
23.14	0.33	1.5	No good match
23.41	20	89	Heneicosane
23.68	1.0	4.5	Tricosene
25.21	3.8	17	Tricosane
25.55	0.95	4.2	Unspecified alkene
25.79	1.1	4.9	Methyl dehydroabioetate
26.05	4.8	22	Tetracosane
26.89	3.8	17	Pentacosane
27.20	2.0	8.8	Di(2-ethylhexyl) phthalate
27.72	3.5	15	Hexacosane
28.68	2.4	11	Heptacosane
29.83	1.6	7.3	Octacosane
31.14	1.2	5.4	Nonacosane
	128	**575**	**SUM ALL**

ANNEX

THE USE OF SHORT- TERM BIOLOGICAL TESTS FOR THE SAFETY ASSESSMENT OF PAPER AND BOARD USED IN FOOD PACKAGING

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1. Introduction

In this chapter the toxicological screening methods that could potentially be applied to paper and board food packaging materials are briefly outlined. The chapter starts with an overview of the present regulatory status regarding the safety testing of fibre-based packaging materials within the European Union (EU). This forms a background for the perceived need for toxicological screening tests and for the general requirements that such tests should fulfil. A number of presently known short term tests are evaluated in the light of these criteria. The few known instances, where short term toxicological screening tests have been applied to paper and board are reviewed. Ongoing research in this area is briefly presented, as well as the impact of the research on the emerging European legislation. Finally some relevant research institutes, available web sites and other additional information are listed for further contacts.

2. Regulatory background

The central directive covering the food contact materials until 2004 was the Framework Directive 89/109/EEC, which was subsequently replaced by Regulation 1935/2004/EC. As a regulation 193572004 became immediately a binding law in all the member states after its publication in the Official Journal of the European Union. The regulation mainly defines the authorization procedures for food contact materials.

Even the Directive 89/109/EEC maintains the general safety provision of the Framework Directive 89/109/EEC which states "*Materials and articles must be manufactured in compliance with good manufacturing practice so that, under their normal or foreseeable conditions of use, they do not transfer their constituents into foodstuffs in quantities which could either endanger human health or bring about an unacceptable change in the composition of the foodstuffs or deterioration in the organoleptic characteristics thereof*". While specific directives exist to address the safety aspects of plastics, ceramics and regenerated cellulose there is, as yet, no specific directive for paper and board intended for food contact. It should be noted, however, that so far there has been no indication of actual harmful consequences associated with food packages consisting of these materials.

2.1. The present regulatory status of paper and board based packaging materials

Paper and board are natural materials, which have a remarkably long history of safe use. Consequently there has been no great pressure to apply specific regulatory measures to ensure their harmlessness in various applications, although in some countries (i.e. France, Italy and Germany) the legislation and the guidelines directed to the industry are rather detailed, especially in the case of recycled fibres (Escabasse and Ottenio, 2002). Typically the existing regulations define the

chemicals that are allowed in the manufacture of paper and board and set limits for various contaminants (heavy metals, pentachlorophenol, polychlorinated biphenyls, etc) in the products. A specific concern of the use of recycled fibre is also reflected in the Council of Europe policy statement on paper and board for food contact (see Section 2.2.1 below).

2.2. The Council of Europe policy statement concerning paper and board materials for food contact

In the absence of specific EU directives, the Council of Europe (CoE) policy statement on paper and board materials and articles intended for food contact remains, so far, the most authoritative document guiding the regulatory practices in Europe (although, as noted above, more specific national regulations already exist in certain countries). The version of the statement published in December 2002 (Anonymous, 2002) contains the specific resolution on the subject, urging the member states to take into account in their national laws and regulations the principles defined in the document. It is specifically stated that paper and board should not transfer their constituents to foodstuffs in quantities which could endanger human health or cause an unacceptable change or organoleptic deterioration. Further, good manufacturing practice is required, the microbiological quality should be guaranteed, paper and board should not release antimicrobial substances, and specific limits for cadmium, led and mercury, as well as for pentachlorophenol are defined. Instructions on testing conditions and on good manufacturing practice are given in specific technical documents attached to the policy statement. Aspects on the use of recycled fibres in the food contact material are given special attention in a separate technical document, the content of which is described in more detail below.

2.2.1. The Council of Europe guidelines on the use of recycled paper and board, limits for harmful substances

In the CoE guideline it is stated that some additional requirements are needed to ensure the safety of food contact materials and articles made of recycled fibre, due to the presence of printing inks, adhesives and other substances in the starting material. Aspects that should be considered include the source of recovered paper and board, the processing technologies applied to remove contaminants and the intended use of the product.

The recovered paper and board which is not considered suitable for use as raw materials include waste paper and board from hospitals, paper and board that has been in contact with garbage, stained sacks that have contained chemicals or foodstuffs, certain covering materials, carbonless

copy paper, certain types of household waste paper (used kitchen towels, handkerchiefs etc) and PCB-containing materials.

Specific requirements for the end products include tests and migration limits for various types of toxic or harmful compounds such as Michler's ketone (4,4' bis(dimethylamino)benzophenone), 4,4' bis(diethylamino) benzophenone (DEAB), diisopropylnaphtalenes (DIPNs) phthalates, solvents, partially hydrogenated terphenyls (HTTP), azo colourants, fluorescent whitening agents, primary aromatic amines, polycyclic aromatic hydrocarbons (PAH) and benzophenone. The amounts of these substances either should be below the detection limits or, in some cases (DIPN, HTTP, solvents) as low as can be reasonably achieved. For benzophenone a specific migration limit of 0.1 mg/dm^2 is defined. The requirements generally apply to products intended to be used in contact with aqueous and/or fatty foodstuffs or also with dry, non-fatty foodstuffs (requirements for DIPNs, HTTP, phthalates and solvents).

3. The perceived need for toxicological testing

While there is no actual indication of major risks for public health due to fibre-based packaging materials, the increased safety consciousness of both consumers and legislators may lead to situations, in which streamlining the regulatory framework on the safety of paper and board products, whether based on virgin or recycled fibre, becomes actual. In the present regulations the emphasis is on the chemical analysis of eventual contaminants and to some degree on the microbiological quality, and no requirements of toxicological safety testing apparently exist, yet. However, the potential usefulness of such tests has been indicated in various documents (Escabasse and Ottenio, 2002; the CoE resolution cited below).

The CoE resolution, when defining the end-product requirements for food contact materials made from recycled fibre, makes the following statement: *"Chemical or toxicological screening tests for possible unknown toxic substances are desirable. However, at present the implementation of chemical screening tests for unknown substances might not be feasible. Furthermore, the knowledge about the applicability of toxicological screening tests for paper and board is insufficient for the time being although it should be noted that studies are in progress to establish the validity of these tests for paper and board. The use of these chemical or toxicological screening ´tests on paper and board should be evaluated and should be recommended in the future where necessary, based on new developments and results in this field."*

In the following sections some requirements for toxicological screening tests applicable for paper and board are outlined, the presently available tests are briefly reviewed, and some examples of the actual applications of toxicological test to paper and board are given (Section 5).

3.1. The types of toxicological tests and test conditions required

The toxicological tests required for routine screening of food contact materials should, naturally, have endpoints relevant to consumer safety. In addition they should be cheap, not labour intensive, and easy to perform for a large number of samples. They should also be validated and recognised by regulatory bodies. These criteria automatically exclude traditional animal testing either for acute, subacute or chronic toxicity, and also most of the presently known short term or in vitro tests fall short of fulfilling all of them. However, some experience of either individual short term tests or their combinations to assay extracts of paper and board is starting to emerge (Section 5).

An additional aspect that should be considered is the preparation of samples for testing. Extraction methods should take into account the types of food (aqueous, dry, fatty) with which the paper and board would interact in real life situations, as well as such factors as the duration and the temperature conditions of the contact. The selection of the food simulants used for extraction can also be critical. While most of the biological tests are compatible with water extracts, solvents like ethanol or iso-octane are regularly used in immigration tests for the extraction of lipophilic compounds. These are usually not very well tolerated by the various biological systems and cell types used in in vitro tests. This limits the amounts of extractants that can reasonably be tested, and increases the need of solvent controls in the assays. Change of solvents or concentrating the samples by evaporation may lead to problems with solubility, loss of volatile compounds, and unforeseen chemical interactions. Thus, the development of realistic extraction methods, compatible with the tests systems used, should proceed in parallel with the eventual choice of biological tests for toxicological screening.

4. Presently available short term toxicological tests

Several types of in vitro tests are routinely applied to study the harmful effects at either cellular level (cytotoxicity tests) or on the genetic material (genotoxicity tests). For research purposes the cytotoxicity tests have been valuable screening tools, and in certain cases they can give valuable information on the structure-function relationships and mechanisms of toxicity. However, so far their use in regulatory toxicology has been relatively limited, the emphasis still being on the whole animal studies. Several genotoxicity tests, on the other hand have been thoroughly standardized

validated and included in various recommended guidelines for toxicity testing (i.e. OECD Guidelines on Genetic Toxicology Testing, 471- 486).

4.1. Cytotoxicity tests

In general, permanent mammalian cell lines of variable sources are utilised in the most common cytotoxicity tests. Although there are variations in the details of the test protocols, the cell cultures are usually directly exposed to the test agent in the growth medium, and after a certain exposure time, the resulting toxicity is measured. The endpoints can be simply cell death or growth inhibition, which can be detected by various methods, such as measuring the total protein content (TPC), using differential staining (i.e. neutral red uptake, NRU) or following the ability of the exposed culture to grow and form cellular colonies (CFA). These tests can be used to screen both pure chemicals and complex mixtures isolated from food or various environmental samples (Stammati et al, 1999; von Wright et al. 1992).

In addition to measuring cell death and growth inhibition, which usually do not give much information about the mechanisms of toxicity, cytotoxicity tests with a clear targeted function can sometimes be used. An example is the use of MTT (a tetrazolium salt), which stains blue because of the reaction with the mitochondrial enzyme succinate dehydrogenase. Thus the assay is very sensitive to mitochondrial poisons, although it can be used to measure also general cytotoxicity (Mosmann 1983).

4.1.1. Tests for sublethal toxic effects

Cell death is a very drastic endpoint usually preceded by various other deleterious effects in the cell. With certain types of short term tests it is possible to detect some of these effects, and thus gain information of non-lethal toxicity, which, however, could be relevant for the safety aspects. These tests can be based on certain enzymatic activities or other specific targeted functions in the cell. Some examples are presented below.

When hepatic cell lines that have retained their ability to respond to foreign compounds by the induction of specific drug metabolizing enzymes (cytochrome P450 variants, such as CYP1A1) are used, this enzymatic activity can be measured and used as an indicator of toxicity (Sanderson et al. 1996, Koistinen et al. 1998).

RNA-synthesis is a basic function of living cells, and its rate can be influenced by various factors. Measurement of the cellular RNA-synthesis rate after exposure to the test agent by following the incorporation of radio labelled uridine provides an indication of the toxicity of the sample (Fauris et al. 1985). The method, originally designed for human HeLa S_3 cells, is particularly useful for water samples, since the cell culture medium can be constituted using the test sample as a base. However, the test can also be used by simply diluting the test agent into the growth medium. The test is used as an official test for bottled drinking water in France

An example of the use of a highly specialised cell type to study targeted toxic effects on the cellular metabolism is the recently developed boar spermatozoon motility inhibition test (Andersson et al., 1998). The motility of a spermatozoon depends on the integrity of mitochondrial functions, and thus the action of toxins affecting the energy metabolism is very rapidly detected as reduction of motility. Other end points that can be measured are plasma membrane integrity, astrodome function, and total cellular ATP and NAD reduction. This test has been particularly useful in the detection of certain types of bacterial toxins from various environmental and food sources.

A bacterial assay, the Photobacterium test, based on the inhibition of light emission of a bioluminescent bacterium *Vibrio fisheri* (ISO-standard 11348-1, 1998), originally developed to test the toxicity of industrial effluents, gives an indication of the effects of the test agent on the oxidative metabolism of the cell. As a bacterial assay its advantages are rapidity and relatively low costs, but naturally the differences between bacterial and mammalian systems make the interpretation of the results even more difficult than with other short term tests.

The availability of recombinant-DNA techniques has made it also possible to design cellular lines or microbial strains with highly specific properties for certain types of toxicity tests. In CALUX-test, designed to respond specifically to dioxins, a recombinant mouse cell line, in which the activity of a luciferase enzyme causing bioluminescence is under a control element responding to AhR-receptor. This receptor is specific to dioxin-like compounds, and when combined with a dioxin it activates a cascade in the cell, leading in this case to induction of luciferase, the activity of which can be measured (Amakura et al, 2003). Another example is the use of recombinant yeast cells in which the β-galactosidase gene is under a control element containing an oestrogen receptor. When the hormone or hormone-like compound reacts with the receptor, the enzyme is activated and the activity can be measured spectrophotometrically (Routledge and Sumpter, 1996).

4.2. Genotoxicity tests

As already pointed out above, genotoxicity tests represent an exceptionally high level of standardisation and official recognition among the short term toxicological tests. This reflects the fact that the target of most genotoxic agents, DNA, is similar in all various life forms, and an agent that affects DNA in a bacterium, is likely to do so in humans, too.

Due to their well-established status good descriptions of the standard tests can be found in various text books and laboratory manuals (i.e. Preston and Hoffman, 2001).
The in vitro genotoxicity tests can be roughly divided into point mutation tests, cytogenetic tests measuring chromosomal anomalies and tests for DNA damage and repair.

4.2.1. Point mutation tests

The best known point mutation test is the Ames Salmonella-assay or Ames test. The assay was developed already in early 70s and later updated by the introduction of novel tester strains (Maron and Ames, 1983). The test is based on a number of Salmonella strains that are histidine auxotrophs, or mutated so that they cannot synthesise histidine and consequently cannot grow without an external source of this amino acid. If the strains are exposed to a mutagenic agent that reverses the original mutation, a revertant colony emerges on a solid test medium devoid of external histidine. The number of these revertant colonies is the measure of the mutagenic potential of the test agent. A mammalian microsome fraction (usually isolated from induced rat liver) is routinely included in the test to mimic the mammalian drug metabolism and activation of certain mutagens.

Point mutation tests have been developed also for cultured mammalian cells (de Marini et al., 1989). These tests are based on the mutational resistance to otherwise cytotoxic agents (i.e. TK or HPRT mutations, conferring resistance to trifluorothymidine and 6-thioguanine, respectively). Compared to Ames test and other bacterial assays they are, however, more laborious and time consuming.

4.2.2 Mammalian cytogenetic tests

In mammalian cytogenetic tests the changes in the chromosome number and structure (as seen in a typical metaphase plate), resulting from genotoxic action, are microscopically monitored (Galloway et al. 1994). The chromosome aberrations include gaps and breakages, deletions and chromatid exchanges. These kinds of analysis can be done both in vitro using cultured cells and in vivo by

exposing experimental animals to the test agent and subsequently collecting suitable cells (peripheral lymphocytes, bone marrow cells) for analysis.

Because of the time and skills needed to analyse metaphase chromosomes, micronucleus tests are increasingly used as a simpler cytogenetic assay. The test is based on the presence of chromosomal fragments or whole chromosomes that have not been incorporated into a daughter nucleus at mitosis. Typically they can be seen as a stained body outside the cell nucleus in an interphase cell. The number of induced micronuclei is a measure of the genotoxic activity of the test agent (Miller et al. 1998). Also this test can be performed both in vitro using cell cultures and in vivo by exposing experimental animals to the test agent and subsequent harvesting and analysis of suitable cells. In in vivo experiments the polychromatic lymphocytes are frequently used, since the micronuclei are naturally very easy to detect against the anucleate background (Hayashi et al. 2000).

4.2.3. Tests for DNA-damage and repair

The uses of bacterial mutants that are deficient in DNA-repair functions and thus particularly sensitive to DNA-damaging agents are routinely used to screen potential mutagens in assays that usually are based on differential killing. If a repair- deficient microbial strain is more sensitive to the lethal effects of the test agent than its repair-proficient but otherwise isogenic control strain, one of the targets of the test agents is probably DNA (Hamasaki et al. 1992).

In mammalian cells the traditional assay for DNA-repair has been the test of unscheduled DNA-repair, in which the repair of damaged DNA is detected by autoradiography after incorporation of radio labelled nucleotides into the newly synthesised DNA at the site of the damage (Madle et al, 1994). A novel mammalian assay that measures directly the DNA damage is the so called Comet assay or single-cell gel electrophoresis. The test is based on the fragmentation of the nuclear DNA as a result of genotoxic action. When a cell is subjected to electrophoresis after exposure to the test agent, the DNA fragments migrate in the electric field and can be seen as a "comet tail" after staining with a fluorescent dye. The test can be applied to hepatocytes retaining their ability to metabolically activate mutagens (Uhl et al, 2000).

5. The application of short term tests to paper and board

So far there have been relatively few published reports on the application of in vitro toxicological tests to extracts of paper and board. The published results relate to the use of photobacterium test,

RNA-synthesis inhibition assay or to a battery of different test systems. The outcomes of these trials are summarised below:

5.1. Paper and board extracts in photobacter assay

Photobacter test has been included in the purity and toxicity assays of fibre-based products (Sipiläinen-Malm et al. 1997, Jokinen et al, 2001). In the latter study samples of pulp and food contact board were systematically evaluated using water extracts obtained from homogenised material. According to the results the light intensity curves were highly repeatable the same samples giving consistently similar responses. However, regarding the toxicity, the results were difficult to interpret, because the samples often produced a pattern of variable photoemission induction and inhibition, depending on the concentration. The main practical result was that each board grade had a typical and highly stable response in the test, and this could be used as a quality control parameter for the stability of the production conditions.

5.2. RNA-synthesis inhibition caused by paper and board water extracts

Fauris et al (1998) made a systematic survey on 6 paper and 15 board samples from different European countries using the RNA-synthesis inhibition test for the toxicity screening. The samples represented both recycled (10 samples) and virgin fibres, among the latter both chemical and mechanical pulps were represented. The recycled fibres represented the four categories according to the CEN 1994 standard (the category A represents raw materials consisting of unprinted or uncoloured paper, the categories B, C and D represent increasing use of printed or coloured raw materials, D being made totally from mixed paper and board of variable origin). In the analysis of water soluble matter and in the preparation of water extracts CEN standard procedures were used. The substances that were analysed from the actual samples included total and organic chlorine, pentachlorophenol, total sulphur and nitrogen, formaldehyde, glyoxal, heavy metals Cd, Pb and Cr, bacterial endotoxins and aflatoxin. GC analysis of the Tenax-absorbed material from the samples was also performed.

According to the results the cytotoxicity of the samples ranged form very high (RNA synthesis rate 17% of the control) to non toxic (RNA-synthesis rate 94%). The same range of toxicities was found in samples representing both recycled products and virgin fibres. Among the latter, the toxic samples (RNA synthesis rate 60% or less of the control) 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 GC-chromatogram.

5.3. Extracts of recycled paper in a toxicological test battery

Binderup et al. (2002) have evaluated three different categories of recycled fibre-based food contact papers in a test battery consisting of a standard 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 (paper A). Paper B represented a product consisting of 40% virgin fibre, 40% recycled material from unprinted newspaper cuttings and 20% de-inked paper from newspapers and magazines. Paper C and D were derived from newspapers and magazines, D being de-inked. The samples were extracted both with 99% ethanol and water following the relevant CEN-standards. The extracts were monitored for migrants using GC-IR-MS or GC-HRMS. The papers were also subjected for microbiological analyses (total aerobic bacteria, aerobic and anaerobic spore formers, *Bacillus cereus/thuringensis*, yeasts and moulds).

In the test applied ethanol extracts showed more toxicity than water extracts and also contained higher amount of material in the chemical analysis. Sample A produced least extractants, and was also least cytotoxic. Among the recycled products the sample C was the most toxic in the cytotoxicity assay. 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 sample C showing the highest positive response, while with samples A and B this activity was non significant. With water extracts a weak positive response was observed with samples B, C and D.

5.4. Genotoxicity of ethanol extracts of selected paper and board samples

Ozaki et al. (2004) have studied both the chemical composition and genotoxicity of ethanol extracts of altogether 28 different paper products intended for food contact and representing both virgin and recycled materials. Altogether 20 different contaminants, including, among others, Michler's ketone, and related benzophenone derivatives, hydoxyphenylpropane compounds, chlorophenols and other chlorinated aromatics, were chemically analysed from the extracts. The genotoxicity test battery included a bacterial *rec*-assay (a differential killing assay using DNA-repair-proficient and repair-deficient *Bacillus subtilis* strains) and Comet assay (see Section 4.2.3 above).

Not surprisingly, recycled papers contained both a wider variety and higher amounts of different chemicals than virgin products. In virgin products benzophenone and bisphenol A were typically detected, although at low amounts. Michler's ketone and related bisphenols were typical for recycled products.

Of the 12 extracts of recycled products nine were positive in the *rec*-assay, while only three of the 16 extracts of virgin materials showed genotoxic activity in this test.
Eight extracts positive in *rec*-assay were also subjected to Comet assay, in which six proved to be positive. Significantly, three of the positive extracts were from virgin material.

When individual compounds identified in the extracts were tested as pure chemicals for genotoxicity and the observed activities were related with the actual concentrations detected in the extracts, it was concluded that the concentrations were too small to explain the genotoxicty of the samples, the actual gentoxic agent(s) remaining thus, so far, unknown.

6. Conclusions and future trends

There is a wide variety of different in vitro tests that could be applied to extracts of food contact paper and board. However, there are few published reports of their use for this purpose, and at present it is not possible to form a consistent picture of their general applicability. The outcomes of the two most comprehensive studies published so far (Fauris, 1998, Binderup 2002, Ozaki et al. 2004) illustrate this point. While all studies agree that the toxicity is correlated with the chemical complexity of the paper and board and apparently not attributable to any single compound alone, the different samples and different test systems applied make these the three studies difficult to compare. While the studies show that in vitro tests can be applied to paper and board products, they also show that application of different tests and testing conditions could lead to a rather different interpretation of the results.

As has been stated in section 3, CoE has recognised the potential value of toxicity tests as a method to ensure the safety of the food contact paper and board. In order to be of use for the consumers, industry and regulators, the proposed tests should be evaluated, standardised and validated. This requires that same paper and board samples would be tested simultaneously using different test systems in order to find out eventual correlations or discrepancies between the results obtained. Also the relevance of positive results should be carefully evaluated in the light of whether they

really reflect any toxicological risks to consumers or whether they can be regarded as artefactual. As pointed out in section 3.1. the proper and realistic sample preparation is also essential for the toxicological testing to be meaningful.

In an ideal case the industry and regulators could have available a selection of validated short-term in vitro tests with sufficient historical background for the safety assessment. This would reduce the need of extensive chemical analysis of the products and would circumvent the practical impossibility of applying traditional toxicological tests routinely to paper and board for food contact. The tests should have relatively broad toxicological endpoints so that many types of harmful compounds could be detected, but the test battery could be complemented with highly specialised tests for specific types of chemical or biological risks (special assays for microbial toxins, endocrine disruptors, hormone-like compounds etc). An additional demand, from practical point of view, is that the set up and performance of at least some of the tests should be feasible in an industrial quality control laboratory, and that a large number of samples could be processed in a relatively short time. The use of the photobacterium test to screen the stability of the products and production conditions (see section 5.1. above) illustrates the point.

In order to answer those needs specified above, a collaborative effort (BIOSAFEPAPER) has been undertaken in the fifth EU framework programme. In this project, scheduled to last until the end of 2005 and coordinated by the University of Kuopio, Finland, nine European research institutes and 16 industrial partners aim at establishing a test battery with relevant toxicological endpoints and allowing a decision-tree approach to ensure the consumer safety. An important aspect of the undertaking is also the development of extraction procedures compatible with the tests and reflecting the real life conditions. As the project is considered as a pre-normative research effort, special emphasis is devoted to the translation of the toxicological data to risk assessment, to the provision of a scientific basis for safety recommendations on fibre-based food contact materials and to the dissemination of the results to various stakeholders (industry, regulatory bodies, consumers). The project has been presented in the 3rd International Symposium on Food Packaging (ILSI, Europe, Barcelona 11/17/2004 - 11/19/2004), and a description of the results obtained by the mid-term evaluation of the project are given in the special issue of the journal Food Additives and Contaminants dedicated to the proceedings of the symposium (Severin et al. 2005). More detailed information on the project and its progress can be seen on the project homepage (http:// www.uku.fi/biosafepaper/).

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