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Test procedures for obtaining representative extracts suitable for reliable *in vitro* toxicity assessment of paper and board intended for food contact

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This paper describes the use of a suite of extraction procedures applicable to the assessment of the *in vitro* toxicity of paper/board samples intended for food-contact applications. The sample is extracted with ethanol, water, or exposed to modified polyphenylene oxide (Tenax®) for fatty, non-fatty and dry food applications, respectively. The water extracts are directly suitable for safety assessment using *in vitro* bioassays. The ethanol extracts of the paper/board and of the exposed Tenax require pre-concentration to give acceptable sensitivity. This is because the *in vitro* bioassays can tolerate only a small percentage of added organic solvent before the solvent itself inhibits. The extraction procedures have been selected such that they mimic the foreseeable conditions of use with foods and that they are also fully compatible with the battery of *in vitro* biological assays for the safety assessment of the total migrate. The application of the extraction protocols is illustrated by the results for one of the many paper/board samples provided by the BIOSAFEPAPER project industrial platform members. The assessment indicated that this sample should not be considered as suitable for use with fatty foodstuffs but was suitable for dry and non-fatty foods. Information subsequently received from the manufacturer revealed that this was a non-food-grade product included in the project to test the capabilities of the bioassay procedures. The selection criteria for the test conditions and the suite of methods developed have been prepared in Comité Européen de Normalisation (CEN) format and is currently being progressed by CEN/TC172 as a European Standard.

Keywords: BIOSAFEPAPER; extraction procedures; paper and board; food contact; in vitro testing; bioassay; migration; hazard identification; toxicity assessment

Introduction

Paper and board are not currently subject to any specific legislation at European Union level, but like all food contact materials they should meet the general requirements laid down in Framework Regulation (EC) No. 1935/2004 (European Commission 2004). Article 3 states that (to paraphrase): 'Materials and articles shall not, under normal or foreseeable conditions of use, transfer their constituents to food in quantities which could endanger human health.' A Resolution specific to paper and board has been issued by the Council of Europe (2002). This contains a listing of substances used along with certain purity requirements, extraction limits or migration limits. Possible contaminants are listed too. This Council of Europe list contains a large number of substances (more than 200) for which toxicological assessments have been made, but an even larger number of substances (more than 500) that have not yet been fully evaluated. Paper and board are natural products made up of a large number of organic molecules and, again, the toxicity of all of these substances, individually and in combination, is not known. Therefore, although migration tests and chemical analysis for known harmful substances can be carried out, they cannot be applied comprehensively to a product with an incompletely defined chemical composition, such as paper and board. In addition, it is a virtually endless task to assess the safety of each substance in turn.

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One complementary approach to safety assessment was investigated within the European Union-funded BIOSAFEPAPER project and this is to consider the toxicity of the overall migrate derived from paper and board. Traditional tests for acute and chronic toxicity are time-consuming, expensive, and have ethical concerns (animal experiments). Moreover, they are very difficult to apply to products like paper and board, and the mixtures that may migrate from them. Instead, the BIOSAFEPAPER project developed and intercalibrated a battery of short-term toxicological tests that are applicable to extracts of paper and board. The toxicological tests and some results have been described by Severin et al. (2005) and Bradley et al. (2008). We describe here the development and application of a suite of extraction procedures applicable to the assessment of the *in vitro* toxicity of paper/board samples intended for food-contact applications.

Materials and methods

Materials

Extraction media

Ethanol was obtained from Fisher (Loughborough, UK) and Tenax TA 60–80 mesh from Chrompack. Water was taken from a normal laboratory de-ionizer.

Other chemicals

Other chemicals were of normal laboratory grade, as described by Bradley et al. (2008).

Paper and board samples

A range of sample types was provided by BIOSAFEPAPER project industrial platform members for use in method development and for the assessment of the bioassay procedures.

Methods

Preparation of cold and hot water extracts

The sample was extracted with water as described in EN645 (cold) (EN645 1994) or in EN647 (hot) (EN647 1994). The extract was sterile-filtered through a membrane filter and stored in a sterilized glass bottle.

Preparation of an ethanol extract

A specimen (200 g) of the paper/board sample was cut into strips and placed into a 2 L glass bottle. Aqueous ethanol (95% v/v, 2 L) was added to submerge the sample fully. The bottle was stoppered and stood at room temperature (approximately 23°C) for 24 h. The extraction solvent was recovered from the sample with minimal mechanical pressing and filtered through a Whatman No. 1 filter paper pre-washed with ethanol. A portion (800 ml) of the extract was placed into a 1 L bottle and without heating it was evaporated just to dryness under a gentle stream of nitrogen. The residue was redissolved in 80 ml ethanol and stored in a 100 ml bottle.

Preparation of a Tenax extract

Preparation of cleaned Tenax. Tenax is conventionally activated by oven heating at approximately 300°C in air, but it breaks down to give a constant background bleed (equivalent to approximately 0.5 mg dm^{-2}) in subsequent ethanol extraction. Since this background bleed may interfere with the toxicity tests, an alternative method of activation was developed. The Tenax was Soxhlet-extracted for 16 h with ethanol. It was then transferred to a wide-necked conical flask, placed under a gentle stream of nitrogen, dried at room temperature and then activated by heating for 16 h at 150°C.

Exposure to Tenax. Twelve 1.5 dm^2 circles of paper/board were prepared using a circular knife or scalpel with a circular template. A specimen was placed into a 14 mm internal diameter glass Petri dish and cleaned Tenax (3 g) was added. The Tenax was smoothed using a spatula to give an even bed covering all the specimen. A further two specimens were placed on top of the Tenax with the food-contact surface in contact, followed by a second portion (3 g) of Tenax. This process was repeated until the twelfth and final specimen rested on top. The result was a stack of the twelve specimens in the glass Petri dish, with the Tenax (six beds of 3 g each) making single-sided contact with the food-contact surface of the paper/board. The dish lid was fitted and the assembly transferred to an oven for exposure according to the time and temperature conditions selected for the intended food use (see later).

Preparation of an ethanol extract of the exposed Tenax. The paper/board specimens were removed using tweezers, allowing the exposed Tenax powder to fall into the dish. Minimal brushing-off was used if the Tenax adhered to the paper/board. The exposed Tenax was placed into a conical flask and ethanol (100 ml) was added. The contents were swirled for a few minutes to extract the Tenax and the powder was then allowed to settle. The extract was decanted and filtered through Whatman No. 1 filter paper pre-washed with ethanol. The extraction was repeated using a second and then a third portion of ethanol (each 100 ml). The combined extracts were concentrated to a volume of 80 ml using a gentle stream of nitrogen.

Preparation of blank control extracts

For each of the extraction procedures, a method blank was prepared for submission to the *in vitro* bioassay tests by using water, 95% ethanol or Tenax as appropriate but with no paper/board sample used.

Storage of extracts

The extracts were submitted to the *in vitro* bioassays as soon as possible after preparation to prevent any chemical or microbiological deterioration. If there was expected to be any delay (more than a few hours), the water extracts were stored refrigerated and then brought to room temperature before the bioassay procedures were conducted to allow any precipitate (that may have formed on cooling) to redissolve. The ethanol extracts (as such or as ethanol extracts of Tenax) were by definition sterile and so were stored at room temperature in the dark.

Characterization of the test extracts

Detailed descriptions of the analytical procedures used are given by Bradley et al. (2008). An appreciation of the procedures is useful in interpreting the results and so they are described in outline here.

GC-MS analysis of the water extracts

A portion of the water extract was fortified with internal standards, 14-methylpentadecanoic acid and cholestanol, evaporated to near dryness then derivatized using *N*,*O-bis*(trimethylsilyl)trifluoro-acetamide (BSTFA). The derivatized extract was dissolved in dichloromethane (1 ml) and analysed by GC-MS using a procedure described by Björklund-Jansson et al. (2002). The GC-MS analysis was operated in full-scan mode (m/z 50–600).

GC-MS analysis of the ethanol extracts

For analysis without derivatization, the internal standards 1,9-dichlorononane and 1-fluorononane were added, the extract was diluted by a factor of ten, and then analysed by GC-MS. For analysis with derivatization, the internal standard hexadecanoic acid was added, the sample was evaporated to dryness, derivatized with BSTFA, and analysed by GC-MS as described above.

GC-MS analysis of the ethanol extracts of the exposed Tenax

The Tenax extracts in an ethanol vehicle were analysed by GC-MS with and without derivatization as described above but without the dilution step.

Toxicological assessment of the test extracts

A battery of short-term toxicological tests was applied to determine the cytotoxicity and genotoxicity of the extract. The detailed descriptions of the tests and their outcomes are described by Bradley et al. (2008). Additional testing was performed to illustrate the effect of the extraction methods on the results obtained and this is described here. Cytotoxicity was assayed using the metabolically competent mouse hepatic cell line Hepa-1 with total protein content (TPC) as toxicological endpoint. The Ames test was used to measure mutagenicity using the tester strain TA98 without metabolic activation.

Discussion

Principles guiding the selection of the extraction media

Three guiding principles were considered in developing the extraction protocols for paper/board samples submitted to bioassay procedures:

- *Identity*: the chemical content of the extract prepared should be related to the chemical migration expected for that paper/board sample in contact with foodstuffs.
- *Compatibility*: the extract should be homogenous, stable, free from particulates and suitable for use in the bioassay procedures.
- *Concentration*: the concentration submitted to bioassay should be no less than the concentration of migrants in foodstuffs.

Identity

The identity of substances migrating from paper/board samples will be dependent on the type of foodstuff with which it comes into contact.

Contact with moist, aqueous, acidic and alcoholic foods. The European Standard hot and cold water extraction procedures (EN645 1994; EN647 1994) were considered to be appropriate (Björklund-Jansson et al. 2002) for paper/board samples described as being intended for contact with moist, aqueous, acidic or alcoholic foods and so they were used.

Contact with fatty foods. There are no equivalent standard methods available for paper/board intended for contact with fatty foods. As recommended in the Council of Europe guidelines, the test conditions established for plastics were considered. Directive 85/572/EEC (European Commission 1985) on plastics describes olive oil as a simulant of fatty foods, but clearly olive oil is not suited for a highly absorbent material such as paper or board.

Directive 82/711/EEC, as amended (European Commission 1982, 1997), describes the use of alternative simulants for fatty foods. These methods have been standardized by the Comité Européen de Normalisation (CEN) (EN1186 2002) and describe the use of 95% (v/v) aqueous ethanol and isooctane. Ethanol is the extraction solvent defined for polar plastics (EN1186 2002) and since paper and board are polar substrates, then ethanol was expected to be the most suitable. To test this assumption, work was carried out within the BIOSAFEPAPER project, described in the final project report (European Union 2006) by testing nine paper and board samples using isooctane and 95% (v/v) aqueous ethanol. In every case, the ethanol solution extracted a higher mass of extractable substances than did isooctane (Table 1).

Considering extraction of specific substances, GC-MS analysis of the extractable substances was performed using methods described by Björklund-Jansson et al. (2002). The results for five of the nine samples are presented in Figure 1. This shows that the extracted substances were mainly wood extractives such as fatty acids, resin acids, fatty alcohols, sugars and sterols, along with other chemicals such as phthalates and hydrocarbon waxes. Figure 1 also illustrates that ethanol extracted a greater quantity of total substances analysable by GC-MS and also extracted a better range of both polar and non-polar substances compared with isooctane. As expected, the distribution of substances was skewed slightly towards polar substances using ethanol (e.g. fatty acids) and was skewed slightly towards non-polar substances using isooctane as the extraction solvent (e.g. wax alkanes). Overall, ethanol gave the best balance and the greater total extractables in every case. Therefore, the conclusion was that paper or board samples intended for contact with fatty foods should be subjected to extraction using 95% (v/v) aqueous ethanol.

Table 1. Total extractables (gravimetric, mg dm^{-2}) from nine typical paper/board samples extracted with isooctane or 95% ethanol.[a]

Sample code number	1	2	3	4	5	6	7	8	9
Isooctane	<0.5	0.9	<0.5	11.3	4.7	0.6	15	20	0.9
95% Ethanol	<0.5	2.4	0.8	39	12	9.3	20	31	2.3

Note: [a]Extracted for 24 h at room temperature (approximately 23°C).

Contact with dry foods. Technical Document No. 2 of the Council of Europe Resolution on paper and board recommends that for dry foods that are listed in Directive 85/572/EEC with no simulant specified, then migration testing should use modified polyphenylene oxide (Tenax). Tenax has been used by others to test paper and board intended for dry foodstuffs (Bradley et al. 2002; Summerfield and Cooper 2001; Sturaro et al. 1994; Boccacci Mariani et al. 1999; Aurela et al. 1999) or intended for use at high temperature (Mountfort et al. 1996). A test method for migration from paper and board using Tenax has been standardized (EN14338 2003).

Compatibility

The extracts must be presented in a test vehicle that is compatible with the biological assays used to asses the

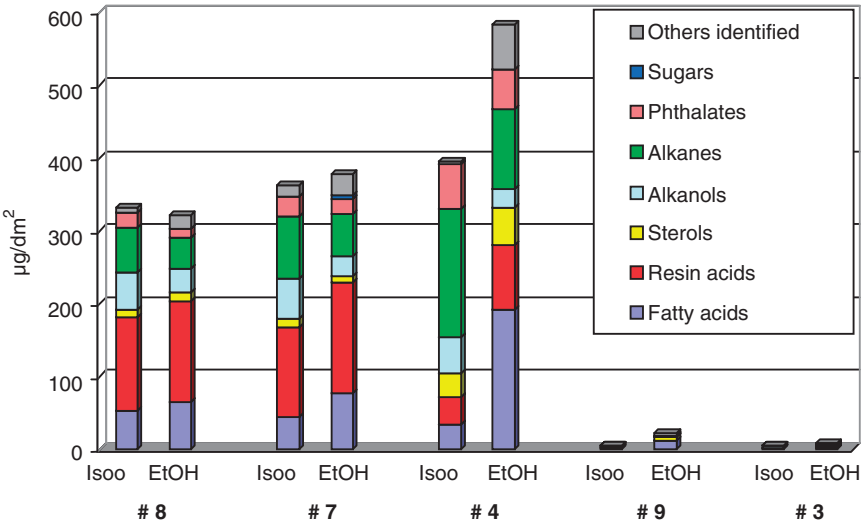

Figure 1. GC-MS comparison of the extractable substances obtained using isooctane and 95% ethanol on five paper/board samples. Extracted for 24 h at room temperature (approximately 23°C).

toxicity of the migrate. The assays that make up the short-term test battery are listed in Table 2.

Aqueous foods. Hot and cold water extracts prepared according to EN645 and EN647 are, following sterile filtration, directly compatible with the *in vitro* assays and so could be used as such.

Fatty foods. The tolerance of the organisms used in the assays to some standard laboratory solvents was determined. The results obtained are given in Table 2 (European Union 2006). Of the solvents tested, ethanol and acetone were considered to be the most suitable delivery vehicles for the extracts. Acetone can become contaminated because it extracts strongly some plasticwares used commonly in *in vitro* assays (data not shown) so ethanol was both the most suitable extractant and the most suitable delivery vehicle for testing paper/board intended for fatty foods.

Dry foods. Tenax is a finely powdered, insoluble polymer. It was necessary to take the exposed Tenax and extract the total migrate from it using a suitable solvent that could then serve to transfer the total migrate into the *in vitro* toxicity test systems. Ethanol is a suitable solvent for extraction of Tenax (e.g. used Tenax was regenerated using ethanol extraction) and is also a suitable vehicle for the toxicity assays (see above) and was therefore selected for use in this way.

Concentration

As mentioned above, the concentration in the extracts tested must be equal to or greater than the levels that would migrate into foods. In general, any tests carried out by total immersion (i.e. the hot water, cold water and ethanol extracts) are more severe than normal or foreseeable conditions of use in contact with foodstuffs and would therefore overestimate any 'real' migration. The extent of the overestimation will depend on the exact food-contact conditions including the time and temperature conditions of use, the nature of the food (i.e. total or point contact, intimate or indirect), the mass-to-area ratio of food-to-packaging, and the chemical composition and physical form of the food. In many cases, the overestimation will be large. The rigorous extraction tests by total immersion will contain the same substances and at higher concentrations than any migration likely to occur into foods. The one significant exception to this is likely to be the extraction of certain heavy metals such as cadmium, chromium and lead, where use of an acidic simulant such as 3% (w/v) aqueous acetic acid gives higher extraction than using plain water (Björklund-Jansson et al. 2002). Since separate purity criteria exist for these undesirable heavy metals (Council of Europe 2002), this is not a significant drawback of the choice of extraction test media selected here.

Tenax is a powdered polymeric simulant and it makes intimate contact with the paper/board samples. Tenax has been shown to overestimate the migration from paper/board samples into foodstuffs (European Union 2003) and therefore it is expected to provide higher migration levels than dry foods. To select the most suitable exposure conditions results obtained within another European Union-funded project (RECYCLABILITY) were considered. Here the kinetics of the migration of model substances from paper/board samples into Tenax were derived. Exposure times and temperatures were selected based on the time taken to reach a migration equilibrium between paper/board samples and Tenax.

As the battery of test organisms can tolerate only up to 2% ethanol in their buffered aqueous culture media, a pre-concentration step is required in order to fulfil the criteria that the concentration of the

Table 2. Maximum solvent concentrations compatible with the battery of short-term cytotoxicity tests (Bradley et al. 2008, European Union 2006).

	Non-toxic concentration in different tests (%, v/v)					
	Acute cytotoxicity tests			Sublethal cytotoxicity tests		
				RNA-synthesis inhibition test		
Solvent	Mouse hepatoma cell line (Hepa-1)	Human larynx carcinoma cell line (Hep-2)	Boar spermatozoan motility inhibition test	HepG2 cells	HeLa cells	Bioluminescence test (EC_{50})
Ethanol	2	1	2	2	0.5	15
Methanol	1	1	2	>2	>0.5	12.5
DMSO	0.5	2	1	2	0.5	>10
Acetone	>2	>2	1	>2	>0.5	7.5
Hexane	>2	Not tested	1	>2	Not tested	0.06

Note: The symbol '>' means that the system tolerated the solvent up to the maximum concentration tested.

substances in the bioassay should be no less than the migration concentration in foodstuffs. This was achieved through the use of a high mass of paper/board to volume of extraction solvent and a further concentration step. The effect of this higher paper/board to solvent ratio on the solubility of the extractable substances and the effect of the concentration step on precipitation problems or loss of volatile extractives were investigated and the optimum extraction conditions were derived (European Union 2006). The optimum ratio found to overcome the solubility limitations was 200 g of paper or board extracted with 2 L of 95% ethanol (i.e. a 1 : 10 w/w ratio). The grammage of the samples described as intended for contact with fatty foods ranged from 140 to 666 g m^{-2} and therefore 200 g of paper/board in 2 L of extraction solvent is equivalent to a range of 0.015–0.071 dm^2 ml^{-1} of ethanol. A further ten-fold concentration step was achieved by evaporation to achieve a final concentration in the range 0.15–0.71 dm^2 ml^{-1}. In EN1186 Part 15 (2002), 1 dm^2 of sample is extracted with 50 ml of solvent giving 0.02 dm^2 ml^{-1}. Therefore, the concentration of the extracts prepared was between 7.5 and 35 times greater than if they had been prepared according to EN1186 Part 15. For further comparison, the hot or cold water extraction procedures (EN645 1994, EN647 1994) use 10 g of paper/board and 200 ml of water (0.04 g ml^{-1}). The concentrated 95% ethanol extract is at a concentration ratio or 1 g paper/board per ml of solvent. This is 25 times more concentrated than the water extracts. Although the high ratio of paper/board to solvent and the evaporation step go some way towards concentrating the extracts, they are not capable of fully accounting for the 50-fold dilution that would be necessary before their use in the bioassays at 2% addition. Further concentration was not possible without loss of migrants due to either solubility limitations or precipitation. As mentioned above, the intimate double-sided contact between the test sample and the extraction solvent provides an extract of much higher concentration than that of the migrate into foods and therefore the concentration factor achieved in this way was considered to be sufficient.

For Tenax, a concentrated sample was achieved by the use of (1) a high ratio of paper/board to Tenax and (2) subsequent concentration of the ethanol extract obtained. The standard method EN14338 (2003) taken as the starting point specifies that 4 g of Tenax is exposed to 1 dm^2 of paper or board and then extracted with solvent to achieve a final volume of 50 ml – giving an extract equivalent to 0.02 dm^2 ml^{-1}. By increasing the ratio four-fold (18 dm^2 tested with 18 g of Tenax) and by concentrating the ethanol extract to a final volume of 80 ml (the minimum volume required for the suite of bioassays) gave an extract equivalent to 0.23 dm^2 ml^{-1} of extract – a ten-times increase in concentration relative to EN14338 (2003). This concentration (on a dm^2 ml^{-1} basis) is within the same range as obtained by the preparation of the ethanol extracts of samples intended for contact with fatty foods (described above). Since Tenax overestimates the migration of paper and board samples into foods (European Union 2003), this concentration was considered to be satisfactory to provide a suitable extract for application in the bioassays in terms of both the identities and concentrations of the migrating substances.

Based on the principles for the selection of the extraction media along with the experimental results obtained, a protocol was derived for the selection of extraction media and extraction/migration conditions. These are summarized in Table 3.

Test results for Sample NSP4 as an illustrative example

Within the BIOSAFEPAPER project, 20 different paper/board samples were tested (European Union 2006). Sample NSP4 was described as 100% recycled Board GD3 (WLC) and its grammage was 300 g m^{-3}. Because its intended use was poorly defined, it was tested using water, ethanol and Tenax. For this reason it is a good example of how the testing scheme elaborated here works in practice.

The concentrated ethanol extract of sample NSP4 showed positive results in genotoxicity assays. The extract induced mutations in the Ames tester strain TA98 without metabolic activation and it was clearly cytotoxic too (Bradley et al. 2008). The extract was analysed by GC-MS as such and also following derivatization with BSTFA. Table 4 lists the substances detected along with their estimated concentrations. The most prominent substances were diisopropylnaphthalene isomers, C15–C29 alkanes and phthalates. Individually, it is not expected that any of these substances would give the positive responses observed in the cytotoxicity or genotoxicity tests. This suggests either that other substances are present in the extract that are not detected by GC-MS and that it is these substances which elicit the positive response or that the response is due to the combined effect of one or more of these substances. Either way, the results emphasize the importance of testing the whole migrate for toxicity as well as considering the individual substances.

Based on these results it was concluded that NSP4 could not be considered as suitable for contact with a fatty foodstuff without further investigations of the cause of the positive results. It later transpired that this sample was a non-food-grade board and therefore this positive response could be used to demonstrate that the battery of tests is capable of detecting a sample that should not be used in contact with a fatty foodstuff.

Table 3. Extraction solvents and test conditions proposed to test paper and board food contact materials.

Food contact conditions	Extraction solvent/food simulant	Test conditions
Contact with moist, aqueous, acidic or alcoholic foodstuffs at temperatures up to 20°C – all times	Cold water	24 h at room temperature (23°C)
Contact with moist, aqueous, acidic or alcoholic foodstuffs at temperatures above 20°C – at all times and temperatures	Hot water	2 h at 80°C
Contact with fatty foodstuffs – at all times and temperatures	95% (v/v) aqueous ethanol	24 h at room temperature (23°C)
Contact with dry foodstuffs – long-term frozen storage	Tenax	10 days at 20°C
Contact with dry foodstuffs – short-term (≤1 week) contact at refrigerated temperature	Tenax	24 h at 20°C
Contact with dry foodstuffs – short-term (≤1 day) contact at ambient temperature	Tenax	24 h at 20°C
Contact with dry foodstuffs – all other contact conditions including high temperature applications but not cooking or baking, or if contact conditions are unknown	Tenax	5 days at 50°C

Table 4. Estimated concentrations (units of $\mu g\,ml^{-1}$ of extract and $mg\,kg^{-1}$ paper) of the substances in the extracts of NSP4.

	Ethanol extract ($\mu g\,ml^{-1}$/$mg\,kg^{-1}$ board)	Dilute ethanol extract ($\mu g\,ml^{-1}$/$mg\,kg^{-1}$ board)	Ethanol extract of Tenax ($\mu g\,ml^{-1}$/$mg\,kg^{-1}$ board)	Substance identification
Is extract cytotoxic?	Yes	No	No	
Is extract genotoxic?	Yes	No	Not tested	
Retention time (min)				
18.16/18.7	218/218	15/256	44/66	Diisopropylnaphthalene isomers
16–29.4	154/154	8.3/138	50/71	C15–C29 *n*-alkanes
20.13	120/120	7.2/119	17/26	Diisobutyl phthalate
25.48	38/38	<LOD	<LOD	Dehydroabietic acid
21.1	37/37	1.7/28	1.5/2.3	Dibutyl phthalate
26.15	29/29	1.9/32	2.0/2.9	*Bis*(2-ethylhexyl) phthalate
19.02	27/27	1.8/31	4.0/5.9	Tetramethyl biphenyl isomer
23.6	22/22	0.76/13	<LOD	*Bis*(2-ethylhexyl) fumarate
22.81	21/21	<LOD	<LOD	9-Octadecenoic acid
24.66	16/16	0.97/16	1.1/1.6	Methyl dehydroabietate
22.48	16/16	1.2/20	<LOD	Octadecenoic acid, methyl ester
23.18	16/16	<LOD	<LOD	Bisphenol A
23.02	6.7/6.7	<LOD	<LOD	Octadecanoic acid
16.25	5.6/5.6	<LOD	<LOD	2-Phenylphenol
25.74	5.1/5.1	<LOD	<LOD	2-(Methoxymethyl)-2-phenyl-1,3-dioxolane
23.14	4.6/4.6	<LOD	<LOD	2-(Phenylmethoxy)naphthalene
17.11	3.1/3.1	<LOD	<LOD	Diethyl phthalate
26.58	2.9/2.9	<LOD	<LOD	7-Oxodehydroabietic acid, methyl ester
23.79	2.6/2.6	<LOD	<LOD	4-Benzyl biphenyl
14.72	2.4/2.4	<LOD	<LOD	Vanillin
17.68	2.4/2.4	<LOD	<LOD	Benzophenone
26.05	2.4/2.4	<LOD	<LOD	Dicyclohexyl phthalate
16.09	1.9/1.9	<LOD	<LOD	No good library match
10.49	1.7/1.7	<LOD	<LOD	Nonanal
6.06	0.6/0.6	<LOD	<LOD	Hexanal
10.54	0.6/0.6	<LOD	<LOD	No good library match
9.29	0.5/0.5	<LOD	<LOD	2-Ethyl-1-hexanol
	757/757	39/653	120/176	Sum
	0.5/0.5	0.5/8.4	0.5/0.7	LOD

Note: LOD, limit of detection.

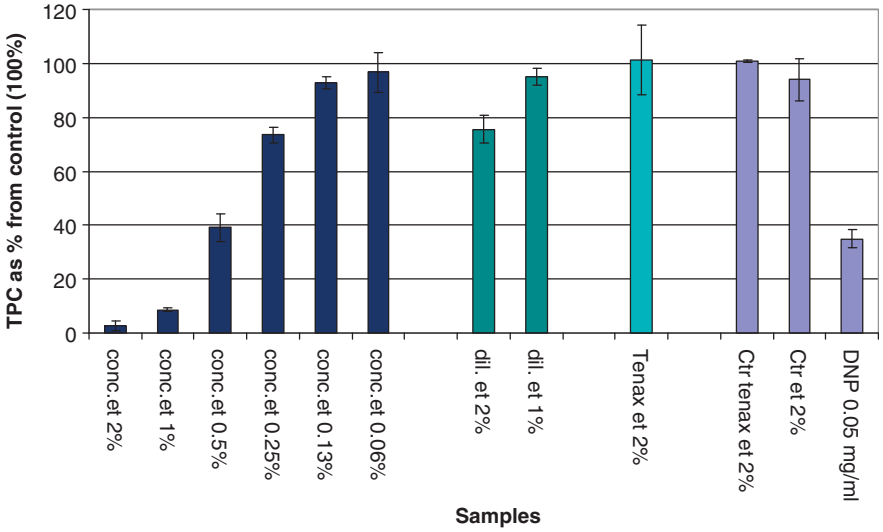

Figure 2. Cytotoxicity of NSP4 against Hepa-1 cells using total protein content (TPC) as toxicological endpoint.
The lower the column, the more toxic is the sample tested.
conc.et, Concentrated ethanol extract; dil.et, dilute ethanol extract; Tenax et, ethanol extract of exposed Tenax; Ctr tenax, ethanol extract of control Tenax; Ctr et, control ethanol; DNP, dinitrophenol, positive control for TPC.

Table 5. Responses of the Ames tester strain TA98 to ethanol extracts of sample NSP4.

Sample	Concentration (μl/plate)	Revertants per plate (± SD) –S9	+S9
Concentrated ethanol extract[a]	0	26 ± 8	32 ± 6
	5	26 ± 6	Not tested
	10	33 ± 2	Not tested
	25	41 ± 6	Not tested
	50	70 ± 17	40 ± 9
	100	90 ± 13	27 ± 8
	200	114 ± 6	43 ± 6
Ethanol extract made according to CEN standard	0	28 ± 1.7	Not tested
	5	25 ± 3.8	Not tested
	10	35 ± 6.1	Not tested
	25	24 ± 1.5	Not tested
	50	28 ± 7.8	Not tested
	100	27 ± 3.6	Not tested
	200	33 ± 2.6	Not tested
Benzo(a)pyrene[b]	2	20 ± 2	235 ± 21
Nitroquinolineoxide[b]	1	589 ± 23	Not tested

Notes: [a]The results of the concentrated extract have been reported separately Bradley et al. 2008.
[b]Positive controls.
CEN, Comité Européen de Normalisation; SD, standard deviation.

A dilute 95% ethanol extract of NSP4 was prepared according to EN1186 Part 15 giving an equivalent of 0.02 dm^2 ml^{-1}. This extract gave no genotoxic response (Table 5) nor any cytotoxic response (Figure 2). This demonstrates that without the concentration step the ethanol extract applied to the battery of toxicological tests is not sufficiently concentrated to assess the safety of the paper/board sample.

This extract prepared without concentration was analysed by GC-MS and the results are compared with those from the concentrated extract in Table 4. The agreement in the results is excellent when expressed in units of mg substance per kg board, both for the individual substances listed and for the total substances detectable by GC-MS, 653 versus 757 mg kg^{-1}.

Further tests were carried out to determine whether or not NSP4 could be considered suitable for contact with aqueous or dry foods. A cold water extract was prepared and the sample was also tested with Tenax for 5 days at 50°C. The extracts were analysed by GC-MS and were tested for cytotoxicity.

The cold water extract of NSP4 contained a number of individual substances. The substances detected (Table 6) were different to those found in the ethanol extracts. This was as expected given the different properties of the two solvents. In addition to the identified substances the GC-MS chromatogram contained many peaks that could not be matched with any library spectra. The total quantity of the non-identifiable peaks was estimated to correspond to approximately 690 μg dm^{-2}.

The range and concentrations of the individual substances migrating from the board into Tenax were less than those extracted by 95% ethanol (Table 4). No cytotoxic responses against the mouse Hep-A strain were observed with the water extract or the Tenax extract (data not shown). Based on these results it can be considered that sample NSP4 is suitable for contact with aqueous and dry foods but not with fatty foods.

Table 6. Estimated concentrations (μg ml^{-1} of extract and mg kg^{-1} paper) of the substances in the water extract of NSP4.

	Water extract (μg ml^{-1}/mg kg^{-1} board)	Substance identification
Is extract cytotoxic?	No	
Is extract genotoxic?	No	
Retention time (min)		
8.01	0.3/7	C9:0 nonanoic acid
8.73	0.1/2	C10:0 decanoic acid
9.93	0.2/6	C12:0 lauric acid
15.95	0.1/2	Resin acid (palustrinic acid)
16.34	0.1/2	Resin acid (not specified)
16.80	0.1/1	Resin acid (not specified)
17.43	0.1/1	Resin acid (isopimaric acid)
17.85	0.2/4	Resin acid (not specified)
18.50	2.2/54	Dehydroabietic acid
19.12	0.2/6	Resin acid (abietic acid)
6.59	0.5/12	3-Hydroxypropanoic acid
8.49	0.3/7	3,4-Di-hydroxybutanoic acid
8.86	0.2/6	Mono-hydroxybutanedioic acid
9.80	0.1/3	4-Hydroxybenzoic acid
10.61	0.2/5	Vanillic acid
32.90	0.2/4	Lignan (conidendrinic acid isomer)
33.13	0.4/11	Lignan (conidendrinic acid isomer)
Various	10/247r	No good library match
	15.5/380	Sum
	$<0.1/<1$	LOD

Note: LOD, limit of detection.

Conclusions

Test methods for the preparation of extracts suitable for *in vitro* toxicological assessment have been elaborated along with the rationale for their selection. The test methods were applied to 20 representative paper and board samples. The results of the chemical characterization and toxicological assessment of one of these 20 samples are presented to illustrate the approach. This board sample was found not to be suitable for contact with a fatty foodstuff. Individually, it is not expected that any of the substances identified in the ethanol extracts would result in the positive responses observed in the cytotoxicity or genotoxicity tests. This suggests either that other toxic substances are present in the extract that are not detected by GC-MS, or that it is the combined effect of two or more of these substances that elicits the positive response. Either way the results emphasize the importance of testing the whole migrate for toxicity as well as considering the individual substances. The selection criteria for the test conditions and the suite of methods developed has been prepared in CEN format and is currently being progressed by CEN/TC172.

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