



## New approach methods for assessing indoor air toxicity

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### ABSTRACT

Indoor air is typically a mixture of many chemicals at low concentrations without any adverse health effects alone, but in mixtures they may cause toxicity and risks to human health. The aim of this study was by using new approach methods to assess the potential toxicity of indoor air condensates. In specific, different *in vitro* test methods including cyto- and immunotoxicity, skin sensitization and endocrine disruption were applied. In addition to biological effects, the indoor air samples were subjected to targeted analysis of 25 volatile organic compounds (VOCs) and Genapol X-80 (a nonionic emulsifier) suspected to be present in the samples, and to a non-targeted “total chemical scan” to find out whether the chemical composition of the samples is associated with the biological effects.

The results confirm that assessing health risks of indoor air by analysing individual chemicals is not an adequate approach: We were not able to detect the VOCs and Genapol X-80 in the indoor air samples, yet, several types of toxicity, namely, cytotoxicity, immunotoxicity, skin sensitization and endocrine disruption were detected. In the non-targeted total chemical scan of the indoor air samples, a larger number of compounds were found in the cytotoxic samples than in the non-cytotoxic samples supporting the biological findings. If only one biological method would be selected for the screening of indoor air quality, THP-1 macrophage/WST-1 assay would best fit for the purpose as it is sensitive and serves as a good representative for different sub-toxic end points, including immunotoxicity, (skin) sensitization and endocrine disruption.

### 1. Introduction

Poor indoor air quality (IAQ) is potentially a greater health risk than outdoor pollution as we spend ca. 90% of time indoors. The main sources for indoor air pollution are emissions from building and cleaning materials, furnishings, electronic equipment, and combustion (burning fuels, tobacco, candles etc.) (Cincinelli and Martellini, 2017; Tran et al., 2020). Poor IAQ has been associated with several detrimental health consequences, including respiratory and cardiovascular diseases, allergy, asthma, headaches, eye and skin irritation, nausea, fatigue and immunological reactions (GIHN 2017; Mendell et al., 2009; Tran et al., 2020; WHO, 2021). Especially in children, it is also a risk factor for neurological diseases and neuropathology (Suades-González et al., 2015), and later in life for the development of e.g. Alzheimer's (Calderón-Garcidueñas et al., 2016) or Parkinson's (Hu et al., 2019) disease.

Currently indoor air investigations focus on chemical, i.e., volatile organic compound (VOC), carbon monoxide (CO), carbon dioxide (CO<sub>2</sub>), ozone (O<sub>3</sub>), nitrogen dioxide (NO<sub>2</sub>), sulfur dioxide (SO<sub>2</sub>), formaldehyde, lead and particulate matter measurements, physical, i.e., humidity, temperature and radon measurements, as well as microbiological investigations (Hess-Kosa, 2018; Tran et al., 2020; WHO, 2021). The assessment of health risks e.g. by analysing the concentrations and effects of individual chemicals is, however, not a valid approach. Firstly, because of the vast number of chemicals present: In 2021 WHO listed 90 compounds that are commonly encountered in the indoor air. The list included VOCs, heavy metals and pesticides, but in reality the list is longer because at least mycotoxins, which are also known to be present in the indoor air (Bennett and Klich, 2003; Nevalainen et al., 2014), and known to cause severe health effects (Ratnaseelan et al., 2018; Vaali et al., 2022), were missing. Hence, measuring the concentrations of all

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chemicals with potential health risks is virtually impossible. Secondly, the amounts of individual substances may be negligible and without any adverse health effects as such (Sundell, 2004; Pistollato et al., 2020), but in mixtures they may cause significant toxicity and risks to human health. Exposure to indoor air should indeed be considered as a chronic exposure to mixture of potentially harmful substances at low concentrations. As also outlined in EU's chemicals strategy for sustainability towards a toxic-free environment (EESC, 2020), there is a big need for methods and strategies to identify the risk of chemical mixtures on human health.

Functional assays measuring health risks from mixtures of chemicals would be very beneficial and needed for adequate risk assessment because substances may co-act in ways such as addition, antagonism, potentiation, and synergy, that affect the overall toxicity. Animal tests (*in vivo*) are not practical and suitable for predicting toxicity of mixtures of environmental origin. Furthermore, to reduce animal testing, the adoption of new approach methodologies (NAM), including *in vitro* or *in silico* methods, are nowadays strongly encouraged (Bal-Price et al., 2018; USEPA 2021). At present, there are a wide variety of biological *in vitro* methods, many of them incorporated as ISO standards or OECD test guidelines (e.g. OECD, 2018; OECD, 2020; ISO 19040-2), for evaluating safety and efficacy of drugs, chemicals, food additives, medical devices and environmental samples such as (waste) waters etc. These are primarily used in biomedical research and testing of single substances but could have potential in indoor air (mixture) monitoring as well.

The investigations on the biological (cellular) effects of indoor air have been performed mainly from particulate matter, either settled dust (Andersson et al., 2010; Huttunen et al., 2015; Hyvönen et al., 2021) or aerosolized particles (Andersson et al., 2010; Tirkkonen et al., 2016; Nordberg et al., 2020; Nordberg, 2022). Dust extracts collected from spaces with poor IAQ have shown to cause immunotoxicity in mouse RAW264.7 macrophages (Huttunen et al., 2015; Tirkkonen et al., 2016), and to inhibit boar sperm motility (Andersson et al., 2010; Salin et al., 2017; Hyvönen et al., 2021). Most recently, particulate matter was shown to activate genes associated with different toxicological pathways, especially immunotoxicity, in a human *in vitro* airway construct (Nordberg et al., 2020; Nordberg, 2022).

In our previous investigations (Hyvönen et al., 2020; Mannerström et al., 2020; Vaali et al., 2022) we introduced a method in which the cytotoxicity of water samples condensed from indoor air was studied in THP-1 macrophages using WST-1 assay (an indicator of mitochondrial activity). The advantage of collecting water samples from indoor air is that it represents indoor air as we inhale it. In addition, it is expected to be a carrier for volatile compounds, but also for high-molecular weight non-volatile compounds, such as cleaning agents (e.g. Genapol X-80), biocides (Selkäinaho et al., 2018) and mycotoxins (Gareis and Gottschalk, 2014; Salo et al., 2019), which may play a major role in causing adverse health effects of indoor air.

The aim of this study was by using several new approach methods, i. e., methods other than traditional animal-based tests, to assess the potential toxicity and health hazard of indoor air (condensates). In specific, we studied the effect of 23–40 indoor air samples on the viability of THP-1 macrophages and monocytes (WST-1 cell viability assay), cytokine secretion (ProcartaPlex™ immunoassay), skin sensitization (DPRA and LuSens) and endocrine disruption (YES/YAS). The main goal was to investigate the validity of WST-1 macrophage assay to predict indoor air hazard by comparing it with the other biological end points. A secondary aim was to assess whether by using the combination of these biological assays it is possible to shed light on the potential health risks of indoor air. Finally, chemical analysis was performed to investigate whether chemical composition of the indoor air samples is associated with the biological effects. For this purpose, the indoor air samples were subjected to targeted analysis of 25 VOCs suspected to be present in the samples, and Genapol X-80. Genapol X-80 is a non-volatile organic compound widely used e.g. in paints and cleaning agents. It represents one example of the many possible non-volatile compounds potentially

present in the indoor air. In addition, a group of samples (cytotoxic and non-cytotoxic) were subjected to non-targeted chemical analysis for a qualitative screening of the relative number of unknown components.

## 2. Materials and methods

All testing was performed in GLP-compliant laboratories. GLP principles were followed when applicable.

### 2.1. Human cells

Human THP-1 monocytes (Cat. No. TIB-202) were purchased from American Type Culture Collection (ATCC, LGC Promochem AB, Borås, Sweden, <https://www.atcc.org>). Prior to experiments, the cells were tested for Mycoplasma (MycAlert™, Lonza, Inc Walkersville, MD, US), and were Mycoplasma-free.

A genetically modified human keratinocyte cell line LuSens was kindly provided by BASF, Germany for research purposes. Prior experiments, the LuSens cell line was tested for Mycoplasma sp. by qPCR with negative results.

### 2.2. Materials

#### 2.2.1. Material for biological analysis

Roswell Park Memorial Institute (RPMI) 1640 Medium was purchased from ATCC (Borås, Sweden). Fetal bovine serum (FBS) and penicillin streptomycin (Pen/Strep) (for THP-1 cells) were purchased from Gibco Invitrogen (Carlsbad, CA, USA). Dulbecco's Modified Eagle's Medium (DMEM), FBS Superior, Pen/Strep (for LuSens), phosphate-buffered saline (PBS) and Trypsin/EDTA were purchased from Biotech Ltd (Cambridge, UK). Phorbol 12-myristate 13-acetate (PMA), nickel (II) sulphate hexahydrate, lipopolysaccharide (LPS), puromycin dihydrochloride, thiazolyl blue tetrazolium bromide (MTT), ethylenediamine-tetra-acetic acid trisodium salt (EDTA), dimethyl sulfoxide (DMSO), acetic acid, ethylene glycol dimethacrylate (EGDMA) and lactic acid (LA) were from Sigma Aldrich (Steinheim, Germany). WST-1 (water soluble tetrazolium salt) cell proliferation reagent was from TAKARA MK400 (Basel, Switzerland). ProcartaPlex™ Immunoassay Human cytokine multiplex was from AH Diagnostics (Helsinki, Finland). Steady-Glo Luciferase Assay System was from Promega. XenoScreen® YES/YAS assay was from Xenometrix (Allschwil, Switzerland).

#### 2.2.2. Material for chemical analyses

A standard of Genapol X-80 (Isotridecyl alcohol polyglycol ether) (code 48750) was purchased from Sigma-Aldrich. LC-MS grade Chromasolv methanol was from Honeywell (Charlotte, NC). Ultrapure water (resistance of 18.2 MΩ/cm) was prepared using the Purelab Classic system (ELGA LabWater, High Wycombe, UK).

### 2.3. Collection of indoor air samples

A total of 40 water samples condensed from indoor air (hereinafter "indoor air samples", "samples" or "condensates") were collected from different facilities in Finland including private homes, public buildings, offices and schools using stainless steel collectors (Aattela, 2020). The sampling was performed as described by Mannerström et al., (2020). Briefly, dry ice (-79 °C) was placed inside the stainless steel collector. Temperature near the collector's surface decreased, and when the dew point was reached the air condensed to liquid water and froze on the collector surface. Frost was melted, trickled on to the receiver, and was then collected into Eppendorf tubes. Temperature and relative humidity (RH%) were recorded. Temperature ranged usually between 20 and 25 °C, and RH% between 25 and 40%. Samples were collected only if the relative humidity (RH%) was at least 25%. The reasons for indoor air sampling were that people were suffering from adverse health effects

and suspected poor IAQ, or there were no health effects but the indoor air quality was to be checked before the building was re-used, or after water damage. Specifying the reason for sampling was voluntary, and a full description of the sampling site and the reason for sampling was obtained from 2 sampling sites only (Sites 1–2, see Table 5). The indoor air samples were sterile-filtered (pore size 0.20 µM), and stored at 4 °C until tested. Before exposure to cells, the samples were warmed to 37 °C.

Due to shortage of sample material not all samples could be tested in all assays. The number of samples tested in each assay is given in Table 1.

#### 2.4. Cell culturing

**THP-1 cells** were grown at 37 °C in humidified air (humidity > 95%) containing 5.0% CO<sub>2</sub>, and were sub-cultured at least twice (but max three times) before seeding for cytotoxicity assays in 96-well plates as follows:

**THP-1 monocytes** were seeded in 96-well plates in RPMI 1640 supplemented with 10% FBS at a density of 10 000 cells/well/50 µl. The cells were grown for 24 hrs prior to exposure.

For activation towards THP-1 macrophages THP-1 cells were seeded in 96-well (for cytotoxicity assay) or 24-well (for cytokine secretion experiment) plates at a density of 100 000 cells/ml in RPMI 1640 supplemented with 10% FBS and 25 nM PMA. The cell numbers were 10 000 cells/well and 100 000 cells/well for 96-well and 24-well plate, respectively. Cells were grown in this activation medium for 48 hrs, after which the activation medium was removed and replaced with RPMI 1640 supplemented with 10% FBS, but no PMA. The medium volumes were 50 µl for 96-well plates and 500 µl for 24-well plates, respectively. Cells were grown 24 hrs prior to exposure.

**The LuSens cells** were cultured in T75 flasks with 20 mL of complete culture medium (DMEM, 10% FBS, 1% Penicilin/Streptomycin, 0.05 µl/ml Puromycin dihydrochloride) at 37 °C in humidified air containing 5.0% CO<sub>2</sub>, and were passaged every 2–3 days. One day prior to exposure, 80–90% confluent cells were harvested and seeded in 96-well plates at a density of 10 000 cells/well/120 µl, and incubated for 24 hrs.

**Saccharomyces cerevisiae strains** provided with the commercially available test kit (XenoScreen YES YAS, Xenometrix®), expressing the human estrogen (hERα) and androgen (hAR) receptors were pre-cultured three days prior to exposure in growth medium supplied with

**Table 1**

Description of biological and chemical analyses used to assess the quality of the indoor air samples (condensates).

Type of the test method and standardization (if any)	Assay	Commercial reagent or kit used	End point	Number of samples analyzed	Performing laboratory	
<b>BIOLOGICAL ANALYSIS</b>	CELL VIABILITY ASSESSMENT	THP-1 macrophage/WST-1 assay (24 hrs exposure)	WST-1 reagent	Measurement of mitochondrial activity as an indicator of cell viability	40	FICAM, Tampere University, Finland
		THP-1 monocyte/WST-1 assay (24 hrs exposure)	WST-1 reagent	Measurement of mitochondrial activity as an indicator of cell viability	23	FICAM, Tampere University, Finland
	IMMUNOTOXICITY	Cytokine secretion by THP-1 macrophages (24 hrs exposure)	ProcartaPlex™ Immunoassay	Measurement of cytokine production as an indicator of immunological activation	30	FICAM, Tampere University, Finland
	SKIN SENSITIZATION OECD TG 442C and 442D	Direct peptide reactivity assay	Peptides (prepared and purified by Centic Biotec)	Measurement of peptide depletion as an indicator of covalent binding/reactivity of chemicals	39	NIPH, Czech Republic
		LuSens (keratinocyte) assay (48 hrs exposure)	One-Glo®, MTT-reagent	Measurement of induction of Keap1-Nrf-ARE signalling pathway of keratinocytes as an indicator of oxidative and/or electrophilic stress.	40	NIPH and Medical Faculty of Palacky University, Czech Republic
	ENDOCRINE DISRUPTION	Yeast estrogen screen (YES/a-YES)	XenoScreen®	Measurement of binding to estrogen receptor as an indicator of estrogenic activity	38	NIPH, Czech Republic
	Yeast androgen screen (YAS/a-YAS)	XenoScreen®	Measurement of binding to androgen receptor as an indicator of androgenic activity	36	NIPH, Czech Republic	
<b>CHEMICAL ANALYSIS</b>	Detection of 25 different VOCs	GC/FID	–	Measurement of concentration of 25 selected VOCs	21	NIPH, Czech Republic
	Detection of Genapol X-80	HPLC/ESI	Infinity 1290 and Poroshell 120 SB-C18	Analysis of Genapol X-80 ( <i>m/z</i> )	21	NIPH, Czech Republic
	Qualitative analysis (total scan)	HPLC-MS/MS	–	Analysis of the relative content and <i>m/z</i> of unknown components	8	NIPH, Czech Republic

Abbreviations and explanations:

ESI: Electrospray ionization source.

FICAM: Finnish Centre for Alternative Methods.

GC/FID: Gas chromatography with flame-ionization detection.

GC/ECD: Gas chromatography with electron capture detection.

HPLC: High pressure liquid chromatography.

LuSens: A keratinocyte based ARE (antioxidant response element) reporter gene assay (for skin sensitization hazard identification).

MS: Mass spectrometry.

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide. A yellow substrate that is cleaved by metabolically active cells to yield a dark blue formazan product.

*m/z*: Ratio of mass and charge.

NIPH: National Institute of Public Health.

THP-1: Tohoku Hospital Pediatrics-1, a human acute monocytic leukemia cell line.

VOC: Volatile organic compound.

WST-1: Water-soluble tetrazolium salt. A red substrate that is cleaved by metabolically active cells to yield a dark red formazan product.

the assay kit on orbital shaker (31 °C) and seeded in 96-well test plates on the day of exposure at a density given in the standard operating procedure provided by the manufacturer (i.e., min. OD<sub>690</sub> of 0.2).

## 2.5. Analysis

The biological and chemical analysis performed are summarized in Table 1.

### 2.5.1. THP-1/WST-1 assay (cell viability)

The exposure of THP-1 monocytes and macrophages to indoor air samples was performed as described by Mannerström et al., (2020). Shortly, the indoor air samples were dosed to cells in cell culture medium supplemented with 5% FBS and 200 IU/ml Pen/Strep at 10% concentration. Each sample was tested in 6 replicates. Cell culture medium with 10% distilled water was used as vehicle (negative) control. Nickel II sulphate hexahydrate, final concentrations 2.0 and 20.0 µg/ml, was used as positive control. The results of indoor air samples were accepted only if the positive control run at the same time with indoor air samples caused > 5% but < 20% loss in THP-1 macrophage viability, and showed dose-dependency. Exposure time was 24 hrs.

After exposure the cell viability was assessed by using WST-1 assay. WST-1 assay is based on the reduction of WST-1 (tetrazolium) salt to formazan, primarily by mitochondrial dehydrogenases of metabolically active cells, and is hence a measure of cell viability, cytotoxicity and proliferation (Mosmann 1983). To perform the assay 10 µl of WST-1 reagent was added to all wells for 3 h at 37 °C, 5% CO<sub>2</sub>. Then the absorbance of the formazan product was read at 450 nm using Tecan Spark plate reader.

### 2.5.2. Cytokine secretion by THP-1 macrophages

For cytokine secretion experiments, THP-1 macrophages were exposed to indoor air samples in cell culture medium supplemented with 5% FBS and 200 IU/ml Penicillin and Streptomycin at 10% concentration. Cell culture medium with 10% distilled water was used as vehicle (negative) control, THP-1 macrophages exposed to 100 ng/ml LPS was used as positive control. After 24 hrs exposure, the cell culture medium from the THP-1 macrophage cultures was collected, and centrifuged for 5 min at 10 000 × g. Cytokines (IFN-gamma, IL-1 beta, IL-3, IL-5, IL-6, IL-8, IL-10, IL-12p70 and TNF-alpha) secreted to the cell culture medium were quantified using ProcartaPlex™ Immunoassay Human Cytokine multiplex kits according to manufacturer's instructions. (Multiplex kit measures simultaneously multiple analytes, in this case cytokines, in a single assay.) 50 µl of each sample and positive and negative control (without dilution) were tested in duplicates. The incubation time for samples was 80 min. The multiplex plates were analyzed using Luminex xMAP technology -based Bio-Plex 200 system (Bio-Rad Laboratories), which is a magnetic bead -based immunoassay in microplate format allowing simultaneous detection of numerous cytokines. Cytokines were tested using two separate multiplex kits, one for SET I samples (10 samples) and another for SET II samples (20 samples).

### 2.5.3. Direct peptide reactivity test for prediction of skin sensitization (DPRA)

Reactivity of test samples with a model hepta-peptide containing cysteine (Ac-RFAACAACOOH) (Gerberick et al., 2004) was used for prediction of skin sensitizing potential according to published DB-ALM Protocol no 154: Direct Peptide Reactivity assay (DPRA) for skin sensitisation testing, with two optimized versions for volumetric approach. In the first version, 50 µl of indoor air samples were diluted in 200 µl of acetonitrile (ACN) resulting in a final volume of 250 µl, i.e. 20% concentration of the sample. Then, 250 µl of the 20% sample in ACN was mixed with 750 µl of a 0.667 mM peptide stock prepared in phosphate buffer (pH 7.5), resulting in a final sample concentration of 5%. [Peptides were prepared and purified by Centic Biotec (Heidelberg, Germany) to > 98.56%]. In the second version, 250 µl of samples as such

were mixed with 750 µl of a 0.667 mM peptide stock in phosphate buffer (pH 7.5), resulting in final sample concentration of 25%. ACN and EtOH were used as vehicle controls (VC). 100 nm Cinnamic aldehyde (CAS 104-55-2) in ACN was used as positive control (PC). Peptide reactivity was reported as peptide depletion based on percent decrease in the concentration of non-reacted peptide in the sample relative to that of vehicle controls (ACN, EtOH). Following incubation, the peptide was quantified by reverse-phase HPLC using an external standard linear calibration curve. The percentage of peptide depletion above 13.8% was evaluated as a positive result.

### 2.5.4. LuSens skin sensitization test

The LuSens assay was performed according to the published DB-ALM protocol (DB-ALM Protocol n° 184: LuSens Assay) with optimization for a volumetric approach (testing of percentage concentrations). Indoor air samples were dissolved in the DMEM with 1% FBS to achieve final concentrations of 25 and 50%. 120 µM EGDMA (positive control) and 500 µM DL-LA (negative control) and vehicle control (DMEM + 1% FBS) were included in each test run. The cells were exposed to the samples for 48 h (37 °C, 5% CO<sub>2</sub>). One-Glo® luciferase substrate (Promega) was used according to the procedure provided by the manufacturer for quantification of the induction of the reporter gene expression (luc) and the luciferase activity was measured by a plate luminescence reader (GLOMAX Multi Reader, Promega). In parallel, MTT viability assay was performed and the resulting formazan concentration was measured at 570 nm. Each sample was tested in duplicate. MTT is a tetrazolium salt which is reduced to formazan by mitochondrial dehydrogenases thus being a measure of cell viability and proliferation (similarly to WST-1).

### 2.5.5. Yeast estrogen / androgen screen (XenoScreen® YES/YAS) (endocrine disruption)

A commercially available yeast-based microplate assay (XenoScreen® YES/YAS) designed for screening estrogenic and androgenic agonistic/antagonistic activities, i.e., endocrine disruption potential, was performed according to the provided standard operating procedure, using the supplied standardized material and chemicals (Xenometrix, 2017) with optimization for a volumetric approach. The purpose was to screen the indoor air samples at the highest possible concentration for the potential of endocrine disruption. One day prior exposure, 50 µl of the indoor air samples in duplicates were applied to the 96-well plates and evaporated under a constant flow of sterile air in a laminar hood. On the day of the assay, the samples were diluted in the reaction solution to the final concentration of 25%. 1% DMSO was used as the solvent control, with respect that the positive controls were also diluted in DMSO, and the final concentration of DMSO was 1%. 17β-estradiol [10–8 M], was used as the positive control in the agonistic (estrogenic) assay. 4-Hydroxytamoxifen [10–6 M] in the antagonistic (anti-estrogenic) assay. 5α-dihydrotestosterone [10–6 M] in the agonistic (androgenic) assay. Flutamide [10–8 M] in the antagonistic (anti-androgenic) assay. The pre-cultured suspensions of two recombinant *Saccharomyces cerevisiae* strains expressing the human estrogen (hERα) and androgen (hAR) receptors were exposed to the reaction solution containing the indoor air samples for 48 h on orbital shaker (31 °C) with semi-humidified atmosphere. All evaluated concentrations were non-cytotoxic to the *S. cerevisiae* cells. The optical density of the red product resulting from conversion of the yellow substrate (CPRG) after secretion of β-galactosidase was measured on BioTek Eon High Performance Microplate Spectrophotometer at 570 nm. The OD<sub>570</sub> of the end product in comparison with the response to controls provides direct correlation with the endocrine activity of the test samples.

### 2.5.6. Chemical detection of VOCs

Gas Chromatography with flame-ionization detection (GC/FID) and gas chromatography combined with electron capture detection (GC/ECD) with previous concentration of analytes by the Purge and Trap method were used to analyze the indoor air samples for the presence of

VOCs (Table 2). Principle of the method: VOCs were isolated from water samples with the use of Tekmar 3000 Purge & Trap Concentrator (Purge Trap K VOCARB 3000) by gas extraction, captured on a solid Carboxen B/Carboxen 1000 & 1001 sorbent and then thermally desorbed directly onto a capillary column of the gas chromatograph with FID + ECD detection (Agilent 6890) with VOCOLTM capillary column, 105 m × 0.53 mm × 3.0 µm. Demineralized water was produced by a two-stage Purelab Option; Purelab Ultra device was used to prepare all calibration solutions or to dilute samples. Mixed standard solution (Chromservis, ref.no. C7FD, Chromservis ref. no. 7037), 1,2-dichloroethane 200 µg/ml (Supelco Lot. XA11118V) and MTBE 2000 µg/ml in methanol (Restek Lot. A0119127) were used for calibration.

### 2.5.7. Chemical detection of Genapol X-80

The instrumental analyses of Genapol X-80 were carried out using an Infinity 1290 high-performance liquid chromatograph (Agilent Technologies, Santa Clara, CA) coupled to a 6490A triple-quadrupole mass spectrometer (Agilent Technologies) equipped with an electrospray ionization source (ESI). For screening purposes, 10 representative parent ions were selected and 20 MS/MS transitions were optimized: 835 → 818 (collision energy = 24 eV), 835 → 315 (28), 747 → 730 (20), 747 → 547 (24), 733 → 716 (20), 733 → 547 (24), 659 → 642 (18), 659 → 459 (22), 601 → 584 (16), 601 → 415 (20), 571 → 554 (16), 571 → 371 (20), 513 → 496 (14), 513 → 327 (18), 483 → 465 (10), 483 → 283 (16), 426 → 418 (4), 426 → 318 (10), 394 → 377 (6), 394 → 195 (14). The chromatographic separation was performed using a Poroshell 120 SB-C18 2.7 µm (150 × 3.0 mm) column (Agilent Technologies) maintained at 40 °C. The gradient of the mobile phase consisting of (A) 2 mmol/L of ammonium acetate in demineralized water and (B) methanol was delivered as follows: 0 min, 30% B, 0.30 mL/min; 1 min, 70% B, 0.30 mL/min; 5 min, 90% B, 0.40 mL/min; 7 min, 90% B, 0.40 mL/min.

### 2.5.8. Qualitative chemical analysis ("total scan")

Selected samples exhibiting significant biological activity, i.e., cytotoxicity to THP-1 macrophages in the WST-1 assay, were subjected to a further qualitative chemical analysis using HPLC-MS/MS (Waters Xevo TQ-S micro, ACQUITY H-Class PLUS, Waters) total scan. A positive

**Table 2**  
Volatile organic compounds analysed in the indoor air water condensates.

Compound	CAS	Detector
1,1-dichloroethene	75-35-4	ECD
1,2-dichloroethene	156-60-5	ECD
MTBE	1634-04-4	FID
dichloromethane	75-09-2	ECD
chloroform	67-66-3	ECD
tetrachloromethane	56-23-5	ECD
benzene	71-43-2	FID
1,2-dichloroethane	107-06-2	ECD
trichloroethene	79-01-6	ECD
bromdichloromethane	75-27-4	ECD
toluene	108-88-3	FID
tetrachloroethene	127-18-4	ECD
dibromochloromethane	124-48-1	ECD
chlorobenzene	108-90-7	FID
ethylbenzene	100-41-4	FID
m-xylene	108-38-3	FID
p-xylene	106-42-3	FID
o-xylene	95-47-6	FID
styrene	100-42-5	FID
bromoform	75-25-2	ECD
1,4-dichlorobenzene	106-46-7	ECD
1,2-dichlorobenzene	95-50-1	ECD
1,3,5 - trichlorobenzene	106-46-7	ECD
1,2,4 - trichlorobenzene	120-82-1	ECD
1,2,3 - trichlorobenzene	87-61-6	ECD

Abbreviations:

ECD; electron capture detector.

FID; a flame ionization detector.

ionization mode (ESI<sup>+</sup>) was used, and the range of *m/z* (mass/charge number) was set from 50 to 750. Cytotoxic samples, (No. 20, 18, 5, 19 from SET I, see [Supplementary material 1](#)), were compared to non-cytotoxic samples, (No. 10, 11, 13, 17 from SET I, see [Supplementary material 1](#)) and distilled water (negative control). The relative content and the ratio of mass and charge (*m/z*) of unknown components present in the samples were determined. The results are presented in [Fig. 1](#) and [Fig. 2](#).

## 2.6. Data handling

### 2.6.1. THP-1/WST-1 assay (cell viability)

The absorbance results were normalized, i.e., the viability of the untreated control was always set as 100%, and all other data was calculated relative to the control value. Results were expressed as either % decrease in cell viability (negative values) or % increase in activity (positive values), which reflected increased mitochondrial activity. The statistical significance of the differences between indoor air-sample treated cells and untreated control cells was tested using *t*-test (Sigma-Plot 14.0). When *p* < 0.05, the difference was considered statistically significant.

### 2.6.2. Cytokine secretion by THP-1 macrophages

The concentrations for each cytokine were extrapolated from their own standard curves by Bio-Plex Manager software. Results were given as x-fold changes as compared to untreated vehicle (negative) control. The cytokine levels produced by untreated vehicle cells and cells treated with 100 ng/ml LPS are shown in [Table 3](#). IL-3, IL-8 and IFN gamma were excluded from the calculations because they were not secreted by THP-1 cells (IL-3), or the multiplexes for SET I and SET II samples did not function consistently (see the explanations in [Table 3](#)). Only at least 3-fold changes were considered relevant changes, otherwise the result was "no induction" (see [Supplementary material 1](#)). The requirement for the at least 3-fold change as compared to untreated control was set because each sample was tested only once (although in two duplicates), and the statistical analysis was hence not possible.

### 2.6.3. Skin sensitization (DPRA and LuSens)

LuSens test was performed to complement the results of DPRA, as neither DPRA nor LuSens should be used as a stand-alone test method to predict skin sensitization. In DPRA, the percentage of peptide depletion above 13.8% was evaluated as a positive result. In LuSens, tested samples were considered to show the sensitizing potential if the luciferase induction was ≥ 1.5-fold and the viability was ≥ 70% at all evaluated concentrations compared to the vehicle control. The results of indoor air samples were only accepted if the average luciferase activity induction obtained with the positive control was ≥ 2.5-fold and with the negative control < 1.5-fold as compared to the average value of the vehicle control. In addition, the standard deviation of the vehicle control did not exceed 20%.

### 2.6.4. Yeast estrogen / androgen screen (XenoScreen® YES/YAS) (endocrine disruption)

The increase in the absorbance value ≥ 25% compared to the value of solvent control was predicted as a positive response in the agonistic assays. The decrease in the absorbance value ≥ 25% comparing to the value of solvent control was predicted as potentially positive response in the antagonistic assays, considering a general biological variability of 20% and a precautionary conservative evaluation.

## 3. Results

### 3.1. Biological analysis

In order to verify the ability of THP-1 macrophages to secrete cytokines, the macrophages were exposed for 24 hrs to 100 ng/ml LPS

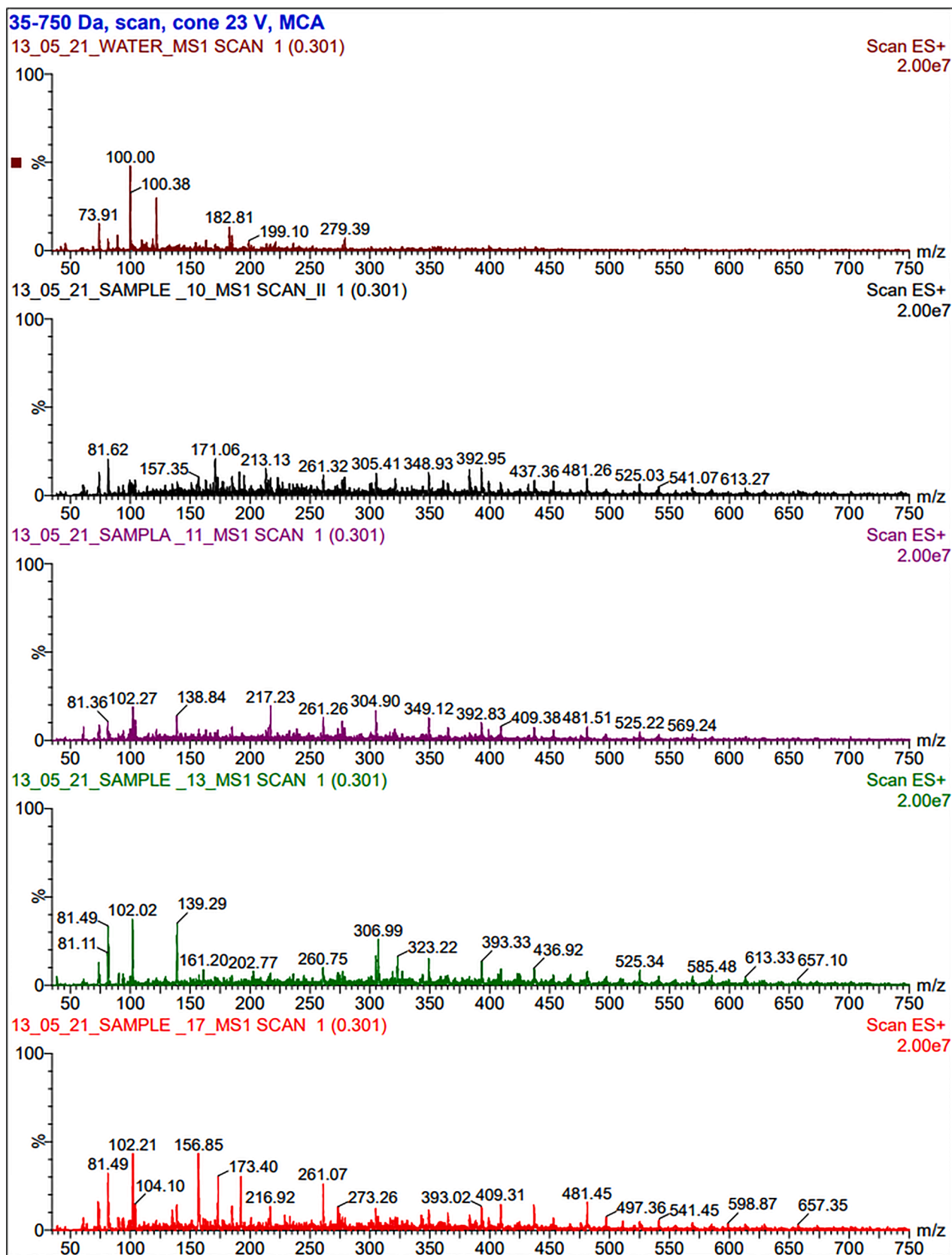


Fig. 1. Detection of unknown compounds in non-cytotoxic (according to THP-1 macrophage/WST-1 assay) indoor air samples using HPLC-MS/MS ("total scan"). Each peak represents a compound having a specific mass-to-charge ratio ( $m/z$ ) and the length of the peak indicates the relative abundance (%) of the compound.

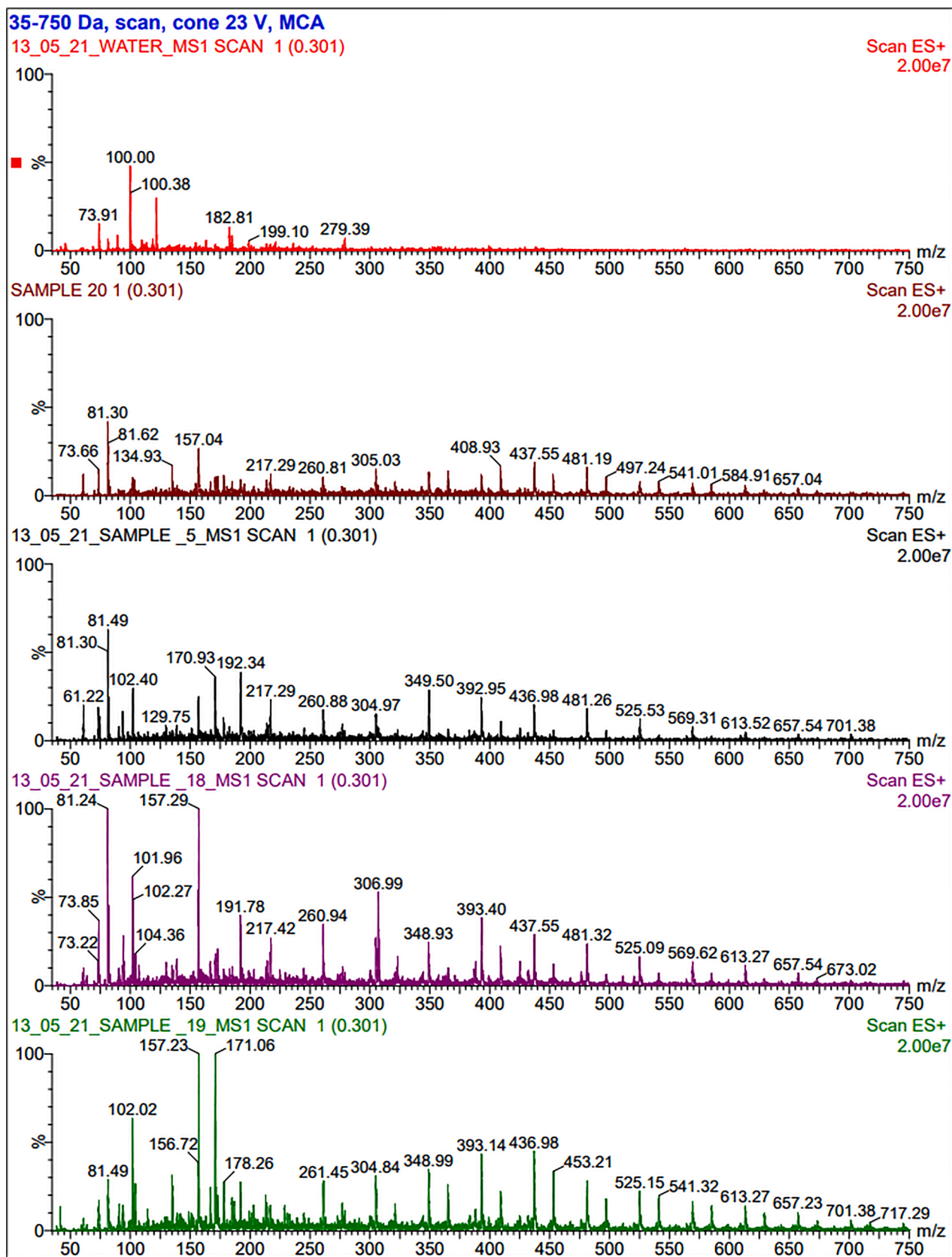


Fig. 2. Detection of unknown compounds in cytotoxic (according to THP-1 macrophage/WST-1 assay) indoor air samples using HPLC-MS/MS (“total scan”). Each peak represents a compound having a specific mass-to-charge ratio ( $m/z$ ) and the length of the peak indicates the relative abundance (%) of the compound.

**Table 3**

Cytokine secretion by untreated THP-1 macrophages (negative/vehicle control) and LPS-treated THP-1 macrophages (positive control). Cytokine experiments were performed using two different Procarta Plex™ multiplexes, one for SET 1 samples, and another for SET 2 samples. IFN- gamma, IL-3 and IL-8 were excluded from the calculations for the reasons as explained in Comments-section.

Cytokine	Cytokine secretion by untreated THP-1 macrophages (VC), pg/ml		Induction of cytokine secretion (pg/ml) by THP-1 macrophages treated with 100 ng/ml LPS (x-fold change as compared to untreated control given in parenthesis)		Comments
	SET I (multiplex 1)	SET II (multiplex 2)	SET I (multiplex 1)	SET II (multiplex 2)	
<b>IL-1 beta</b>	3.90	34,12	280.80 (72.0x)	403.14 (11.80x)	–
<b>IL-5</b>	5.30	5.15	12.58 (2.40x)	74.10 (14.40x)	–
<b>IL-6</b>	2.07	6.23	3832.40 (1851.40x)	2577.10 (413.70x)	–
<b>IL-10</b>	0.41	2.21	14.94 (36.40x)	14.60 (6.60x)	–
<b>IL-12p70</b>	3.85	1.52	9.72 (2.50x)	4.80 (3.16x)	–
<b>TNF alpha</b>	4.33	8.47	4017.40 (927.80x)	2008.60 (237.10x)	–
<b>IFN gamma</b>	3.23	1.20	11.82 (3.66x)	0.72 (0.60x)	IFN gamma did not work on multiplex 2 as shown by the lack of positive control (LPS) to induce it
<b>IL-3</b>	below detection range				IL-3 was not secreted by THP-1 macrophages
<b>IL-8</b>	78.00	845.20	above the range of std curve	1248.40 (1.48x)	The multiplexes (1 and 2) were inconsistent; in multiplex 1 the signals were above the range, in multiplex 2 positive control (LPS) did not induce IL-8 secretion

(positive control), which is a glycolipid of the outer membrane of Gram-negative bacteria, and known to induce strong inflammatory response. The secretion of cytokines by untreated THP-1 macrophages (vehicle/negative controls) and LPS-treated THP-1 macrophages is presented in Table 3. Detection of IL-1 beta, IL-6, IL-10, TNF alpha, IL-12p70 and IL-5 was consistent in the two multiplexes used as shown by the positive control. IL-3, IL-8 and IFN gamma, in turn, were excluded from the calculations for following reasons. IL-3 was not secreted by THP-1

macrophages (neither in negative nor in positive controls). Further, the results of IL-8 were not consistent as in one of the multiplexes its secretion was above the standard curve, and in the other it was below the standard curve. IFN gamma secretion by positive control, in turn, was detected in the first multiplex, but not in the second. This suggests that there were differences in the sensitivity of certain cytokines in the two multiplexes, whereas other cytokines showed good reproducibility.

Table 4 summarizes the results of all biological assays. For detailed

**Table 4**

Summary of the biological analyses of the indoor air samples. The three most sensitive assays to detect adverse effects in indoor air samples are bolded.

Assay	Adverse effect detected	Number of samples causing adverse effect	Number of samples causing no effect	Proportion of adverse effects from all samples	Controls
<b>THP-1 macrophage/WST-1 assay</b>	Inhibition of mitochondrial activity as an index of cell viability	32	8	<b>80%</b>	NC: Distilled water PC: Nickel Sulphate Hexahydrate
THP-1 monocyte/WST-1 assay	Increased mitochondrial activity as an index of monocytosis	4	19	17%	NC: Distilled water PC: Nickel Sulphate Hexahydrate
Cytokine secretion by THP-1 macrophages	Increased cytokine secretion (IL-1 $\beta$ , IL-5, IL-6, IL-10, IL-12 p70 and/or TNF- $\alpha$ ) as an index of immunological activation	12	18	40%	NC: Distilled water PC: Lipopolysaccharide
<b>Direct peptide reactivity assay</b>	Covalent binding to peptide as an index of reactivity/electrophilicity of the sample	26	13	<b>67%</b>	NC: Acetonitrile and EtOH PC: Cinnamic aldehyde
LuSens (keratocyte) assay	Induction of Keap1-Nrf1-ARE pathway as an index of oxidative/electrophilic stress	8	32	20%	NC: DL-LA PC: EGDMA
Yeast estrogen screen (YES/a-YES)	Binding to estrogen receptor as an index of estrogenic activity	10	28	26%	SC: 1% DMSOPC: 17 $\beta$ -estradiol (agonistic) and 4-Hydroxytamoxifen (antagonistic)
Yeast androgen screen (YAS/a-YAS)	Binding to androgen receptor as an index of androgenic activity	12	24	33%	VC: 1% DMSOPC: 5 $\alpha$ -dihydrotestosterone (agonistic) and Flutamide (antagonistic)
<b>YES/a-YES or YAS/a-YAS effect</b>	Endocrine disruption	20	18	<b>53%</b>	see above
All assays combined	Any of above mentioned effects, or several of them	38	2	95%	

Abbreviations:

DL-LA: Lactic acid.

DMSO: Dimethyl sulfoxide.

EGDMA: Ethylene glycol dimethacrylate.

NC: Negative control.

PC: Positive control.

SC: Solvent control.



results, see [Supplementary material 1](#). Thirty-two of 40 samples tested (80%) were cytotoxic to THP-1 macrophages in the WST-1 assay. The (statistically significant) inhibition in THP-1 macrophage viability ranged from 3.40% to 11.00%. Of the skin sensitization assays, 26 of 39 samples (67%) were DPRA positive, and 8 of 40 samples (20%) exhibited a potential of skin sensitization in LuSens. 20 of 38 (53%) exhibited endocrine activity, 10 of these showing antiestrogenic effect, 12 showing androgenic effect, and two of the samples being both antiestrogenic and androgenic. None of the samples were estrogenic or antiandrogenic. Twelve samples of 30 (40%) induced cytokine secretion (immunotoxicity). The induced cytokine secretion was typically 3–10-fold as compared to control, except for one sample (No 5, SET I, see [Supplementary material 1](#)), where IL-1beta secretion was 129-fold, IL-6 secretion 1411-fold, IL-10 42-fold and TNF-alpha 392-fold. Four samples of 23 (17%) increased the mitochondrial activity of monocytes (ranging from 18.10% to 35.40%), and this was always accompanied by increased proinflammatory cytokine, especially TNF- $\alpha$ , secretion. No cytotoxicity (decreased mitochondrial activity) was observed in THP-1 monocytes.

Of the 32 samples that were cytotoxic to THP-1 macrophages, 30 caused also other adverse effects in one or more of the endpoints studied, i.e., they were (skin) sensitizers, caused cytokines secretion, were endocrine disruptors and/or induced monocytosis (proliferation of THP-1 monocytes). Of the 8 samples that were not cytotoxic to THP-1 macrophages, 6 caused adverse effects on some of the other endpoints studied. Two samples of the total 40 samples caused no adverse effects whatsoever.

Description of sampling sites and reasons for sampling are given in [Supplementary material 1](#). Out of the 24 indoor air samples from sites where people reported adverse health effects, 22 (92%) were cytotoxic to THP-1 macrophages. A full description of the sampling site and the reason for sampling was obtained for 2 sites. The connection between health effects and adverse biological findings in these sites are presented in [Table 5](#).

### 3.2. Chemical analysis

The results from the chemical analysis are summarized in [Table 6](#).

#### 3.2.1. Detection of VOCs

No increased level of VOCs was confirmed in any of the samples. All the values of the monitored VOCs were found to be below the limit of quantification (0.1  $\mu\text{g}/\text{l}$ ). The chromatographic profile of the samples was similar to that of the blanks (data not shown).

**Table 5**

The association between sampling site, reported health effects and the adverse effects detected in the biological methods.

	Description of the sampling site and health effects	Summary of observed results
Site 1Samples No. 11 and 12 (SET II) <sup>1</sup>	Office building. Damp and smelly. Many health symptoms reported, e.g. asthma, recurrent respiratory infections, neurological symptoms, elevated blood pressure	Toxic to THP-1 macrophages Induced IL-5 and IL-12p70 secretion, Skin sensitization markers DPRA and LuSens positive Androgenic, anti-estrogenic
Site 2Samples No. 1 and 2 (SET I) <sup>1</sup>	Office building. Employers suffer from several health effects including recurrent infections, migraine, severe fatigue, sore throat, gastrointestinal problems and irritation of eyes	Toxic to THP-1 macrophages Induced THP-1 monocyte proliferation (monocytosis) Induced IL-1 beta, IL-10 and TNF alpha secretion Skin sensitization markers DPRA and LuSens positive

<sup>1</sup> See [Supplementary material 1](#).

**Table 6**

Summary of the chemical analyses of the indoor air samples.

Analysis	Result
Detection of 25 different VOCs	GC/FID GC/ECD VOCs were not detected in any of the samples
Detection of Genapol X-80	HPLC/ ESI Genapol X-80 was not detected in any of the samples
Qualitative analysis (total scan)	HPLC- MS/MS Higher peaks of unknown components with higher frequency were detected in cytotoxic samples as compared to non-cytotoxic samples (see <a href="#">Fig. 1</a> )

#### 3.2.2. Detection of Genapol X-80

After introduction into the ESI interface operated in positive polarity mode, Genapol X-80 standard solution provided a rich mixture of chemicals with the main peak at  $m/z$  of 571, and approximately 80 signals at  $\geq 5\%$  relative intensity distributed between  $m/z$  of 282 and the end of the monitored range,  $m/z$  of 1000. Under the conditions used, all monitored Genapol X-80 signals co-eluted between 4.8 and 5.3 min but were readily distinguished on the basis of the selective MS/MS signals. For this reason, it was impossible to perform a quantitative determination of Genapol X-80 as a sole individual chemical. Therefore, a qualitative screen to detect the presence of most probable components of Genapol X-80 was performed. From the full ESI-MS spectrum, 10 spectral peaks with good intensity distributed between  $m/z$  394 and 835, representing markers of Genapol X-80 that were selected. These markers were monitored during the HPLC-MS/MS run of each indoor air sample. None of the Genapol X-80 marker signals was detected in the indoor air samples. The limits of detection (LODs) of the target Genapol X-80 congeners ranged between 5 and 20 ng/ml. For instance, the most intense transition 571  $\rightarrow$  554 provided a signal-to-noise ratio of 8 at LOD of 5 ng/ml.

#### 3.2.3. Qualitative analysis ("total scan")

Samples (20, 19, 18, 5 from SET I, see [Supplementary material 1](#)) which exhibited most cytotoxicity to WST-1 macrophages in the WST-1 assay, were selected for further qualitative analysis in comparison to samples which were not cytotoxic (samples 10, 11, 13, 17 from SET I, see [Supplementary material 1](#)) and to negative control (distilled water). The non-targeted HPLC-MS/MS analysis ("total scan") was performed in order to screen the relative content, the frequency of peaks and the ratio of mass and charge ( $m/z$ ) of unknown components present in the samples. Significantly higher peaks [over 50%] of unknown components with higher frequency were detected in the selected cytotoxic samples as compared to negative control (distilled water) and non-cytotoxic samples, as demonstrated in [Fig. 1](#) and [Fig. 2](#).

## 4. Discussion

In this study we present a novel strategy for evaluation of indoor air toxicity, which consists of collecting water samples from the indoor air by condensing, and then testing the effect of the condensed water samples using new approach methodologies (NAM). NAM means an approach to provide information on chemical hazard relying on non-animal methods ([Bal-Price et al., 2018](#); [USEPA 2021](#)). The end points studied here covered cyto- and immunotoxicity, hormonal effects and skin sensitization, which can be regarded as predominant effects in case any reactive substances are present in the indoor air. The biological methods were then supplemented with chemical analysis of 25 selected VOCs and Genapol X-80 (representing one example of non-volatile organic compounds) suspected to be present in the samples, as well as non-targeted qualitative chemical analysis.

### 4.1. Indoor air sampling

The advantage of collecting water condensates from indoor air for

toxicity testing as compared to e.g. dust (settled in the course of time) is that they represent indoor air at the moment as we breathe and are otherwise exposed to it. Secondly, it appears that a large variety of indoor air pollutants, including non-volatile compounds (MW > 300 g/mol) are present in the indoor air condensate, as demonstrated by the non-targeted qualitative chemical analysis (see Fig. 2).

Despite of being a polar molecule as a monomer, at room temperatures, i.e., 20–30 °C, water molecules form large clusters with average size ranging from 57 to 47 molecules (Némethy and Scheraga, 1962). In the water liquid/vapor interfaces the hydrated excess protons of these water clusters can effectively display both hydrophobic and hydrophilic character (Petersen et al., 2004), and hence can serve as a carrier for both hydrophobic and hydrophilic indoor air pollutants. There is evidence that various fungi emit mycotoxins (MW > 500 g/mol) into liquid (guttation) droplets, (Gareis and Gottschalk, 2014; Salo et al., 2019), which get aerosolized and migrate through air, which in turn may lead to their exposure via inhalation. Similarly, biocides and Genapol X-80 (MW 552 g/mol) have been shown to mobilize into humid air via aerosolization (Selkänaho et al., 2018). More than 100 volatile, semi-volatile and low-volatile anthropogenic substances were demonstrated in samples collected from the condensate of HVAC (heating, ventilation and air conditioning) systems (Roll et al., 2015). These included several endocrine disruptors such as parabens (MW 152–194 g/mol), Triclosan (MW 290 g/mol), Bisphenol A (MW 228 g/mol) and tetrabromobisphenol A (MW 544 g/mol) (Roll et al., 2015).

#### 4.2. Assessment of indoor air toxicity by biological methods

For the assessment of mixture toxicity several different mathematical models of additivity exist (Rider et al., 2018; EFSA, 2019). There is evidence that sub-toxic concentrations of individual chemicals produce adverse effects in mixtures: No observed effect levels of formaldehyde and acrolein caused cytotoxicity as a mixture (Zhang et al., 2018). Moreover, chemicals representing different classes, i.e., industrial chemicals, pesticides, endocrine disruptors, drugs and persistent organic pollutants, at non-cytotoxic concentration as such may become developmental neurotoxins in a mixture (Pistollato et al., 2020). Mixtures of unknown composition can be assumed to follow the same principles, they act jointly to produce combination effects, i.e., additive effects, synergism or antagonism. Testing the overall effects of indoor air samples on different biological end points represents a whole mixture approach of a sample of unknown composition.

##### 4.2.1. Cell viability and immunological activation

Monocytes and macrophages are essential components of the innate immune system, responsible for defending against diverse pathogens and contaminants (Parihar et al., 2010). Alveolar macrophages are a major population of immune cells in the respiratory tract and the first defense against inhaled pollutants. Here, human THP-1 monocytes as such and as differentiated towards macrophages, were used to assess cyto- and immunotoxicity. Previously we showed that THP-1 macrophages are more sensitive and reproducible to predict the cytotoxicity of indoor air condensates than e.g. human fibroblasts or human bronchial epithelial cells (Mannerström et al., 2020). The reason lies in macrophages being the front line immunoreactive cellular barrier against xenobiotics, getting more exposed to foreign material than other cell types due to the efficient uptake by phagocytosis (Gordon, 2016). Among the biological end-points investigated here, including immunotoxicity, skin sensitization and endocrine disruption, THP-1 macrophage/WST-1 assay was the most sensitive; 32 samples of 40 were cytotoxic (decreased mitochondrial activity) in this assay.

None of the sub-toxic endpoints alone identified adverse effects as comprehensively as THP-1 macrophage/WST-1 assay even if they can be considered warning signals that usually precede toxicity. However, when the outcome of all the sub-toxic methods were put together, in total of 30 of the samples that were toxic in THP-1 macrophage/WST-1

assay exhibited adverse effect(s) also in another biological endpoint, i.e., immunotoxicity, skin sensitization and/or endocrine disruption. Inversely, two of the samples that were cytotoxic to THP-1 macrophages, did not exhibit any other adverse effects. The cytotoxicity of these sample could have been caused by mechanisms not covered here, such as DNA damage or endoplasmic reticulum stress. Given the small (although statistically significant) cytotoxicity (max 11% inhibition in mitochondrial activity) measured in THP-1 macrophages, the association of THP-1 macrophage cytotoxicity with other adverse effects in 30 cases of 32 was an important proof to support the validity of the results obtained with THP-1 macrophage assay.

One explanation for the sensitivity of THP-1 macrophage/WST-1 assay is that (when culturing strictly under standardized conditions paying attention to the susceptibility of THP-1 cells to differentiate prematurely due to changes in pH or excessive cell density), the THP-1 macrophage culture is very stable, and shows minor well-to-well variation in the assay. This enables measurement of small, but statistically significant, changes in THP-1 macrophage viability. The downside of an overly sensitive method would be overestimation of adverse effects, i.e. occurrence of false positives. In this study, only 2 samples were toxic without any other adverse effects. This suggests that false positives are not a concern.

The effects of indoor air samples on THP-1 monocytes showed great variation, which resulted in lack of statistically significant results in most cases. This internal variation may be due to the nature of monocytes being highly reactive and sensitive to environmental changes (Yang et al., 2014). Unlike in THP-1 macrophages, where the effect caused by indoor air samples was cytotoxicity (decrease in mitochondrial activity), in THP-1 monocytes the indoor air samples always caused an increase in mitochondrial activity (up to 35%). This may be due to increased respiration as many microbe toxins are mitochondrial uncouplers (Wallace and Starkov, 2000). On the other hand, increased mitochondrial activity can reflect increased cell number, i.e., monocytosis, which is a marker of several inflammatory diseases *in vivo* (Yang et al., 2014). Interestingly, increased mitochondrial activation of THP-1 monocytes was always associated with induced secretion of pro-inflammatory cytokine TNF-alpha by THP-1 macrophages. Hence THP-1 monocytosis could be considered as an indicator of immunological activation.

To date, >300 cytokines, chemokines and growth factors have been described with varying functions not just on the immune system but on every organ of the body (Turner et al., 2014). Cytokines can be related to a wide variety of health problems, including inflammation, chronic diseases and even neurological diseases. The cytokines investigated here were pro-inflammatory cytokines IL-1 beta, IL-6, TNF-alpha, IL-12p70, anti-inflammatory cytokine IL-10, and IL-5, which is a cytokine associated with allergy and asthma (Dougan et al., 2019; Pelaia et al., 2019). IL-5 is generally not considered to be secreted by macrophages. Unexpectedly, it was produced by THP-1 macrophages after stimuli to LPS (Table 3) as well as after exposure to two of the indoor air samples. Secretion of IL-5 by macrophages gets support from Xu et al., (2018) who demonstrated that CARD9 can turn alveolar macrophages into IL-5 producing cells. Altogether 12 samples (of 30 samples investigated) evoked cytokine secretion. Induction of TNF alpha was the most common effect caused by 7 (of 30) indoor air samples. This is consistent with previous studies showing that components present in indoor air such as particulate matter (Huttunen et al., 2015; Tirkkonen et al., 2016; Glencross et al., 2020), mycotoxins (Rosenblum Lichtenstein et al., 2015; Glencross et al., 2020), and e.g. Bisphenol A (Liu et al., 2014) stimulate pro-inflammatory cytokines, especially TNF-alpha. Furthermore, screening for transcripts has also shown that immunotoxicity is a major cause of toxicity caused by indoor particulate matter (Nordberg et al., 2020, Nordberg, 2022). The induction of cytokine secretion by indoor air pollutants and its health risk should not be overlooked as relatively minor but chronic inflammatory conditions can set the stage for more serious diseases such as neurological diseases, intestinal

diseases, type 1 and type 2 diabetes, obesity and asthma (Turner et al., 2014).

Sample No. 5 (SET I) (see [Supplementary material 1](#)) was clearly distinguishable from the other samples in terms of the levels of cytokine secretion as the inflammatory cytokine secretion was severalfold higher as compared to any other sample. The sample was collected from the basement floor of a house with recent water damage. Therefore, we suspect it contained significant amounts of mold whose metabolic products caused a cytokine storm.

#### 4.2.2. Skin sensitization

Additional immunotoxic end point, as studied using a peptide-based *in chemico* method DPRA (OECD TG 442C) and *in vitro* method LuSens (OECD TG 442D), evaluated the potential of indoor air samples to act as skin sensitizers. The DPRA represents the first key event in the skin sensitization process, i.e., covalent binding of the test item to the peptide resulting in cysteine depletion. 26 of 40 indoor air samples were positive in DPRA assay, but only 8 of them were positive in LuSens, i.e., induced the next key step in the skin sensitization process, which is the activation of Keap1-Nrf1-ARE signalling pathway, one of the most important defense mechanisms against oxidative and/or electrophilic stress. Electrophilic binding to proteins (represented by DPRA) is the first step in both skin and respiratory sensitization (Enoch et al. 2009; 2010), but ultimately the substances that cause skin sensitization are not necessarily respiratory sensitizers, and vice versa. Our data showed best concordance between DPRA test and THP-1 macrophage/WST-1 assay, which make us suggest that the samples that were positive in DPRA but not in LuSens, possess probably respiratory sensitizing hazard. To date no adequate methods are available for distinguishing between skin and respiratory sensitization (Dik et al., 2016; Kimber et al., 2018). Why so many indoor air samples were DPRA positive is alarming but not in itself surprising, considering the vast range of compounds present in indoor air. One typical DPRA-inducer is formaldehyde (OECD TG 442C), which is commonly present in indoor as well as outdoor environments (Salthammer et al., 2010).

An interesting observation in LuSens assay was the large number of indoor air samples (18 out of 40) that increased human keratinocyte (LuSens) viability, as demonstrated by an induction of mitochondrial activity in the MTT assay (see [Supplementary material 1](#)). Proliferation of keratinocytes gives rise to cells that differentiate to produce the epidermal layers. It is a complex process involving several growth factors (Eckert et al., 2002), susceptible for modulation by chemicals (Soroka et al., 2008; Zasada and Budzisz, 2019). Among other chemicals, indoor air contains glycolic acid (Nazaroff and Weschler, 2020) derived e.g. from plastics, solvents, emulsion polymers, additives of paint, which has been shown to induce keratinocyte proliferation (Denda et al., 2010).

#### 4.2.3. Endocrine disruption

Endocrine disruptors are hormonally active, i.e., endocrine disrupting agents. The fact that half of the samples collected from indoor air caused endocrine disruption is reasonable given that there are many possible sources of endocrine disruptors in the indoor air environments: Many consumer products contain Triclosan, Genapol X-80 and Bisphenol A, which are all endocrine disruptors (Goodman et al., 2018; Wazir and Mokbel, 2019; Tachachartvanich et al., 2020). Furthermore, there is evidence that Triclosan and Bisphenol A are carried by indoor air humidity as they accumulate in indoor air condensate (Roll et al., 2015). Genapol X-80 is also suspected to be present in indoor air humidity (Selkainaho et al., 2018).

#### 4.3. Chemical analyses

Chemical analyses were performed to get an idea of the chemical composition present in the indoor air, and their potential association with biological effects. Genapol X-80 was one target of analysis because

it was suspected to be present in the indoor air samples as it is a non-ionic emulsifier widely used e.g. in laundry and cleaning products. However, it could not be confirmed to be present in the samples. Similarly, no VOCs were detected in any of the samples. The VOCs may have been lost during handling and transportation of the samples. In addition, although Genapol X-80 gets mobilized into water vapour and travels with air (Selkainaho et al., 2018), it may spread along floors because it is heavier than air, and could therefore have been missed in the sampling that was performed at a height of ca. one meter above floor level.

The “total scan” analysis demonstrated that higher peaks of unknown components were repeatedly detected with higher frequency in the cytotoxic samples compared to negative control (distilled water) and the non-cytotoxic samples. These results demonstrate the extensive complexity of assessing the safety of indoor air condensates. Albeit biological activity of the indoor air samples was associated with higher peaks and higher frequency of peaks in the “total scan”, it is not possible to find direct association of the biological activity with a particular chemical substance. Therefore, in the case of such complex samples, it is appropriate to consider them as unknown mixtures and to monitor their overall toxicological potential.

#### 4.4. Health effects

Of the sites where people experienced health problems, 92% turned out to be toxic in the THP-1 macrophage/WST-1 assay. The association between health effects and cytotoxicity thus appears significant, but should be treated with caution because the health effects cannot be linked unambiguously to the sampling site as people usually spend time in different spaces (home and workplace/school). Secondly, the information on the sampling sites and reasons for sampling were usually quite general, and we did not get detailed information on what kind of health effects were experienced, nor had we clinical evidence to support them.

For two buildings (Site 1 and Site 2, [Table 5](#)), the health effects were more specifically described: Site 1 was damp and smelly, and several health effects including asthma, respiratory tract infections and neurological symptoms were reported. Several biological adverse effects were measured as well including cytotoxicity to THP-1 macrophages, induced IL-5 and IL-12p70 secretion, skin sensitization (DPRA and LuSens), and endocrine disruption. The health symptoms experienced, especially asthma and neurological symptoms are typical for mycotoxin exposure (Ratnaseelan et al. 2018; Vaali et al. 2022). In turn, the induction of IL-5 is associated with asthma (Dougan et al., 2019; Pelaia et al., 2019), and the induction of IL-12 p70 with the presence of mycoplasma (Rosenblum Lichtenstein et al., 2015). Taken together, the health symptoms and measured biological effects suggest (at least) the presence of mycotoxins in the building.

Similarly, in Site 2, the reported health effects appeared to be related to the diversity of measured biological adverse effects. Health effects included recurrent respiratory infections, fatigue, migraine, and gastrointestinal problems. The biological findings, in turn, were cytotoxicity to THP-1 macrophages, induction of pro-inflammatory (IL-1beta, TNF-alpha) and anti-inflammatory (IL-10) cytokines, as well as monocytosis and skin sensitization.

Although these preliminary results suggest an association between health symptoms and biological adverse effects caused by the indoor air samples, more data is needed to confirm this. This as well as the potential IL-12p70 as a possible biomarker for moisture-damaged and mycotoxin-contaminated buildings, would be an interesting target for future studies.

It should be noted that the tested samples were usually collected from buildings where poor indoor air was suspected, which explains the large number of adverse effects determined in the biological methods, and the low number of samples without any adverse biological effects. A weakness of this study is the lack of reference building with pure indoor air. On the other hand, defining what is pure air is practically impossible

as so many factors, biological, chemical and physical, affect the quality of the indoor air.

## 5. Conclusions

Indoor air is typically a mixture of many chemicals at low concentrations (even below detection limit) without any adverse health effects alone, but in mixtures they may cause significant toxicity and risks to human health. This study utilizing new approach methods confirms that assessing toxicity of indoor air by analysing individual chemicals is not an adequate approach: We were not able to detect the defined VOCs and Genapol X-80 (suspected to be present) in the indoor air samples, yet, several adverse biological effects as cytotoxicity, immunotoxicity, skin sensitization and endocrine disruption were identified. In the non-targeted total chemical scan of the indoor air samples, a larger number of (unknown) compounds were found in the cytotoxic samples than in the non-cytotoxic samples further supporting the validity of the biological methods. If only one biological method (of the method used here) would be selected for the screening of indoor air quality as a routine, THP-1 macrophage/WST-1 assay would best fit for the purpose as it is the most sensitive and serves as a good “representative” for different sub-toxic end points, including immunotoxicity, (skin) sensitization and endocrine disruption. In general, it can be stated that the new approach methodologies are particularly suitable for studying the toxicity of mixtures, such as indoor air, that contain several different compounds in small concentrations.

## CRedit authorship contribution statement

**Mannerström Marika:** Conceptualization, Investigation, Formal analysis, Writing – original draft. **Dvorakova Marketa:** Conceptualization, Investigation, Formal analysis, Writing – original draft. **Svobodova Lada:** Investigation, Writing – review & editing. **Rucki Marian:** Investigation, Writing – review & editing. **Kotal Filip:** Investigation, Writing – review & editing. **Vavrouš Adam:** Investigation, Writing – review & editing. **Vrbíková Věra:** Investigation, Writing – review & editing. **Kejlova Kristina:** Investigation, Writing – review & editing. **Jirova Dagmar:** Writing – review & editing, Supervision, Funding acquisition. **Heinonen Tuula:** Writing – review & editing, Project administration, Funding acquisition.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

The data that has been used is confidential.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.crtox.2022.100090>.

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