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Modeling and MANOVA studies on toxicity and endocrine potential of packaging materials exposed to different extraction schemes

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Abstract: The stability of the linings of packaging that is in contact with the goods stored has been of major concern during decades of the development of packaging materials. In this work, an attempt was undertaken to assess the applicability of using two bioassays (Microtox® and XenoScreen YES/YAS) in estimating the stability of packaging (cans, caps, multilayer material) and the impact of their degradation on the toxicity of some simulated media. The assessment of the impact of packaging storage conditions (temperature, disinfection, preservation, extracting and washing solvents) was planned and performed with i) regression modeling of the experimental effects on the ecotoxicity readings, ii) ANOVA and MANOVA estimation of the experimental conditions as significant factors affecting the toxicity results and iii)FTIR analysis of the packages. It is shown that the effects of temperature and extraction solvents could be quantitatively assessed by the agreement between all methods applied. It can be stated that temperature and acidity as well as the alcohol content in the sensitive media have the greatest impact on the toxicity of the extract and thus on the stability of the internal lining and the extractability of xenobiotics.

Key words: toxicity, endocrine potential, packaging materials, extraction, experimental design, modeling, MANOVA, FTIR

Highlights:

- Simultaneous assessment of the impact of treatment conditions on packages ecotoxicity.
- Checking of the impact of temperature and extracting solvents on endocrine potential of packages.
- Comparison of the package lining spectra before and after extraction.
- Ecotoxicity parameters as a tool in packages lining stability assessment.

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1. Introduction

The introduction of packaging materials has revolutionized the sales of common products. The use of packaging has mainly contributed to the extension of the expiration date of food products and made their transport and storage easier. Currently, a vast majority of products are sold in such packaging as plastics, paper, glass, metal and composite materials Despite the clear benefits of these materials, their long-term use has shown some negative aspects. Since the 1980s, we have known that they might be an additional source of exposing people to xenobiotics (Lau and Wong, 2000). The issue of food packaging safety is regulated by both the EU and domestic legislation. Nevertheless, numerous studies have proven that the small-molecule components of packaging may be eluted from the internal layer of a material due to the presence of the medium stored (Arvanitoyannis and Kotsanopoulos, 2014; Ossberger, 2015). These facts have caused the issues related to the migration of xenobiotics and factors that influence the intensification of the process to become one of the leading topics of interest for researchers. Actions are being taken mainly to identify and quantitatively determine the compounds released and eventually to assess the risk to humans (Canellas et al., 2015; Guart et al., 2011). As can be easily concluded, this task is particularly difficult. Due to a vast number of various types of substances used for the production of packaging materials, there are hundreds of compounds that can migrate from food contact materials to food or food simulated media additives starting from substances such as plasticizers, antioxidants, light and thermal stabilizers, slip compounds, antistatic agents, lubricants, and monomers to heavy metals and nanoparticles (García et al., 2006; Guart et al., 2011; Raptopoulou et al., 2014; Sanches-Silva et al., 2009). It is obvious that the exposure to pollutants always has an unfavorable impact on an organism. However, the threat is particularly severe in this case. A significant number of chemical substances from the abovementioned groups (e.g., bisphenol A, bisphenol A diglycidyl ether and its derivatives, phthalates, primary aromatic amines, perfluorinated compounds and some heavy metals) are classified as endocrine-disrupting compounds (EDC) (Moreira et al., 2013; Moreta and Tena, 2014; Pérez-Palacios et al., 2012; Pezo et al., 2012). The results of numerous studies indicate that EDCs may alter the activity of natural steroid hormones by modifying their regulatory pathways, interacting with steroid receptors and antagonizing endogenous hormones or simply mimicking steroid hormone-dependent effects (Kudłak et al., 2015).

Although first mentions about the activity of these compounds appeared many years ago, they are still the object of great interest for the researchers. More and more advanced techniques used in the research provide novel information about harmful activity of these compounds. The danger is much more significant than it was believed before. Therefore, it was necessary to implement numerous amendments to the regulations concerning acceptable safe doses specified as regards individual substances. The first regulatory standard for bisphenol A was established in 1988 and the oral reference dose was assessed on 50 mg/kg/day. Then the value was reduced to 4 mg/kg of body mass (Ćwiek-Ludwicka, 2015). Regulations related to the acceptable limits of compounds migrating from the surface of a package have also been tightened up. SML value for bisphenol A was reduced in 2018 from 0.6 mg to 0.05 mg/kg of food or food simulant (Commission Regulation (EU) 2018/213). Supposedly, these values will be reduced again in the nearest future.

Additional problem connected with the phenomena of releasing compounds from the surface of the package relies on the fact that apart from well-known starting substances, impurities known as Non-Intentionally Added Substances (NIAS) can be present in food as well as their transformation products. The difficulties related to the identification of all the released compounds and the lack of knowledge about the toxicity of those compounds have resulted in an increase in the importance of using bioanalytical techniques in such research areas (Maisanaba et al., 2014; Ozaki et al., 2004; Wagner and Oehlmann, 2011). The main advantage of *in vitro* bioassays is the possibility of specifying the actual influence of the overall migration, considering most of the interactions occurring between pollutants released. This is particularly important in consideration of the fact that we currently know that compounds coexisting in the mixture may interact and cause an increase (synergism) or decrease (antagonism) in the final effect. There are many premises that such interactions occur between compounds released from the surface of packaging materials (Hu et al., 2014; Li et al., 2017; Wieczerzak et al., 2016). In our previous research, synergistic interactions were found in mixtures that contain such compounds as BADGE·H_2O, BADGE·$2H_2O$ or BFDGE (Szczepańska et al., 2018).

Due to the properties of compounds released, researchers usually apply tests that enable specifying the hormonal activity of the packaging samples. The XenoScreen YES/YAS, ERa and AR CALUX served as analytical tools that enabled the confirmation that the compounds released from some carton and polypropylene packaging show estrogenic activity (YES EEQ = 59.6±29.3 ng/L) (Mertl et al., 2014). Another group of tests used in such studies includes those that provide information on mutagenicity, genotoxicity and cytotoxicity of the given samples (i.e., extracts of the packaging). Based on the results obtained with the use of the rec-assay and comet assay, it was possible to show that the compounds released from the surface of paper and paperboard packaging cause weak genotoxic and cytotoxic effect In the literature, there is some additional information on the possibility of using bioassays in the research on paper and plastic packaging (Galotto and Ulloa, 2010; Ozaki et al., 2005). To the best of our knowledge, there is no information on the toxicity and hormonal activity of compounds released from the internal surface of cans and multilayer composite packaging. Thus, it also seems that the current research will be a significant contribution to present knowledge and that it will be a perfect complement to the existing information on the influence of compounds released from common packaging materials on living organisms. The main objective of the research was the evaluation (with MANOVA data treatment) of the toxicity and endocrine potential of the compounds released from packaging using the XenoScreen YES/YAS and Microtox® biological tests.

2. Methodology

2.1. Experimental

Because one of the most commonly used types of food packaging is currently metallic and multi-material composite packaging, cans, multilayered composite packaging and cups were chosen for the study. The subjects of the study were 60 metal cans devoted to fish storage (total volume ca. 0.15 dm^3), 56 paper hot-drink cups (0.20 dm^3 volume) and 60 multilayered composite packaging units commonly used for milk or juice storage (1 dm^3 volume). None of the samples was in contact with food prior to testing, and care was taken to avoid any contamination of the samples during sampling, storage or transport. Two approaches were used in the study. The goal of the first approach was to estimate the toxicity and endocrine potential of compounds released into simulant liquids, whereas the second focused on determining whether and to what extent the exposure to the extraction agents/conditions results in the degradation of the polymer layer. Fig. 1 shows the schematic approach applied in studies. None of the samples were in contact with food prior to testing, and care was taken to avoid any contamination of the samples during sampling, storage or transport.

Fig. 1.

2.1. Chemicals and reagents

Chemicals that were used for preparing simulant media were obtained from the following suppliers: sodium chloride (CAS no. 7440-23-5) (Sigma Aldrich, Germany), dipotassium phosphate (CAS no. 7758-11-4) (Ciech S.A., Poland), calcium chloride (CAS no. 7440-70-2) (Eurochem BGD, Poland), magnesium chloride (CAS no. 7786-30-3), potassium chloride (CAS no. 7440-09-7), potassium carbonate (CAS no. 584-08-7), lactic acid (CAS no. 79-33-4), urea (CAS no. 57-13-6) (POCH S.A., Poland), ammonium hydroxide (25 % w/w) (CAS no. 1336-21-6), acetic acid (AcOH, 35-38% w/w, CAS no. 64-19-7) (Chempur, Poland), distilled water, EDC-Pak cartridge (Merck, Germany). Microtox® kit (2% NaCl, lyophilized *Vibrio fischeri* bacteria, Microtox Diluent, Microtox Acute Reagent, Osmotic Adjusting Solution (OAS), and Reconstitution Solution (RS) were purchased from ModernWater Ltd. (GB). Ethanol (EtOH, CAS no. 64-17-5), dimethyl sulfoxide (DMSO, CAS no. 67-68-5) and Parafilm® were purchased from Sigma-Aldrich (Germany). All reagents were of analytical grade or higher (reagents used for microbiological purposes). Reagents used for XenoScreen YES/YAS were purchased from Xenometrics G. A. (Switzerland). These were: vials containing hERα yeast cells (for YES assay) and hAR yeast cells (for YAS assay) on a filter paper, basal medium, vitamin, L-aspartic acid (CAS no. 56-84-8), L-threonine (CAS no. 72-19-85) and copper sulfate solutions (CAS no. 7758-98-7), CPRG (chlorophenol red-β-D-galactopyranoside) (CAS no. 99792-79-9), vials with 17β-estradiol (CAS no. 50-28-2), 5α-dihydrotestosterone (CAS no. 521-18-6), 4-hydroxytamoxifen (CAS no. 68392-35-8), flutamide (CAS no. 13311-84-7), DMSO (dimethyl sulfoxide) (CAS no. 67-68-5). 96-well plates, gas-permeable plate sealers, culture flasks with gas-permeable filter cap were purchased from GenoPlast Biochemicals (Poland). All reagents were of analytical grade purity or better in case of reagents for microbiological purposes. The instruments and equipment used during the study were: Microtox® 500 from Modern Water Ltd. (GB), electronic multi- and single-channel pipettes from Eppendorf (Germany), NaOH (CAS no. 1310-73-2) and HCl (CAS no. 7647-01-0) (purchased from Avantor Performance Materials S.A. (Poland)), CP411 pH-meter from Metron (Poland), heater of Thermicon P® (type K1253S) Heraeus Instruments (Germany), microwave heating device (Samsung ME 733K (maximum power 1150 W) and shaker type water bath 357 from Elpan Laboratory Instruments (Poland).

2.2. Preparation of simulant liquids

All simulant solutions were prepared using reagents of analytical grade purity. Artificial saliva was prepared in accordance to the guidelines described in the DIN:53160-1:2010-10 standard (NaCl 0.53 g/dm^3, KCl 0.33 g/dm^3, $CaCl_2{\cdot}2H_2O$ 0.15 g/dm^3, $K_2HPO_4{\cdot}3H_2O$ 0.76 g/dm^3, $MgCl_2{\cdot}6H_2O$ 0.17 g/dm^3, K_2CO_3 0.53 g/dm^3, and 1% HCl 0.75 g/dm^3). The pH of the solution was adjusted using a 1% NH_3 solution to a value of 6.8. To minimize the problem of the background EDC pollution, the Milli-Q water was additionally purified by the use of an EDC-Pak cartridge (for removing endocrine-disrupting compounds) at the stage of the preparation of simulation liquids. Additionally, to reduce the risk of contamination of the glassware with organics, an additional step of heating utensils at 450°C for at least 4 hours was applied (Fierens et al., 2012). Simulation liquids were stored at +4 °C prior to performing the extraction process.

2.3. Sample collection

The migration tests were carried out in accordance with the test procedure indicated in the Commission Regulation (EU) No. 10/2011 and PN-EN 1186-1:2005 standard. Considering the fact that the tested packaging is intended for storing a very wide range of products (such as fruits and vegetables, meat products and various beverages), the simulation liquids most commonly used in the migration studies (distilled water, acetic acid solution and ethanol solution) were used in the study (standard procedures, e.g., the EU legislation recommends using 10% ethanol; however, due to the high alcohol content, an unintended elevated toxicity was observed and a lower ethanol content had to be utilized). Moreover, a 5% dimethyl sulfoxide (DMSO) solution and artificial saliva solution were used to check the extraction strength of other solvents (DMSO is considered as a factor reflecting the lipophilic properties of ingredients present in the product stored). The tests were performed using the filling method: the packages were filled with their respective simulation fluids up to 5 mm below the upper edge. Since cans and multilayered composite packaging are intended for the long-term storage of food, they were held at 60°C for 10 days (240h) after filling,. Additionally, in order to determine the effect of temperature on the degree of release of xenobiotics, the different kinds of packaging were also exposed to temperatures of 65°C and 121°C and the tests were also performed for 336h (these results are presented in manuscript). A different procedure was used in the case of research performed with cups. These were filled with boiled solvents up to 5 mm below the upper edge. Additionally, in order to determine the effect of microwave heating on the migration of chemical compounds from the cups, a microwave treatment was carried out. The treatment was stopped when the temperature of the solvents reached 95–100°C. All objects were shaken with orbital movement at 100 revolutions per minute (rpm) throughout the time period. Table 1 provides more detailed information on the conditions and methods applied in the studies.

The extracts tested were stored at -20°C until the biological studies were performed. To learn of possible changes in the surface-layer composition and structure of the materials studied, samples of packaging material (taken before and after the extraction process) were cut into smaller pieces, and surface analyses were performed on them.

Table 1. Information on methods applied in degradation and migration studies.

Type of packaging	Metal cans	Multilayer composite packaging	Cups
Simulant media	Distilled water, 5% ethanol, 3% acetic acid, 5% DMSO, artificial saliva solution		Distilled water, 5% ethanol, 3% acetic acid, 5% DMSO
Conditions	Room temperature	Room temperature	Room temperature
	100°C - 4 h + 60°C - 10 days	60°C - 10 days	filled with hot solvents (95°C -100°C)
	65°C - 30 min	65°C - 30 min	microwave radiation (10 min at 800 W)
	121°C - 30 min	121°C - 30 min	
Sampling at:	12 h, 48 h, 240 h and 336 h		0.5 h, 1 h, 2 h, 6 h, 12 h

2.4. Procedures of bioanalytical tests

Microtox® is an acute toxicity test, used here in order to determine the level of toxicity occurring after a short time period. The selection of this test was based on the fact that microorganisms represent the primary focus in the food chain, and therefore, any adverse changes occurring in them, directly or indirectly, can have an impact on organisms at higher trophic levels. The XenoScreen YES/YAS test used aimed to show whether the compounds released from the packaging exhibited hormonal activity. The use of genetically modified yeast with human receptors made it possible to estimate the effects of xenobiotics on the health of consumers. Although bioanalytical studies are precisely described elsewhere (Szczepanska et al., 2016; Wieczerzak et al., 2016) we present here complete methodologies to provide a full picture of the work performed.

2.4.1. Microtox ® methodology

Acute toxicity was assessed by determining the luminescence inhibition of the marine Gram (-) bacteria of *Vibrio fischeri*, after a 30 min exposure to respective samples. The degree of the reduction of natural light output emitted by the bacteria is proportional to the degree of toxicity of a given sample. pH was adjusted to fall within the 6.5-7.5 range with NaOH and HCl. Acute toxicity was determined by standard protocol using the Microtox® Analyzer Model 500 and serial dilutions. Lyophilized reagent with *Vibrio fischeri* bacteria was hydrated with 1 mL of RS and maintained at 5.5±1.0°C, subsequently 100 µL of 10-fold diluted reconstituted Microtox Reagent bacterial solution and 2 mL of samples were added into the vials. To produce a suitable osmotic pressure (above 2 %) OAS was added to the vial with the highest concentration and proper dilutions were prepared. For quality assurance of proper test run the following parameters according to producers guidelines were assumed: for Microtox® I_0 of bacterial suspension >70 U (chromium sulphate was used as a positive control of bacterial stock suspension test run).

2.4.2. XenoScreen YES/YAS methodology

The test was performed on the basis of instructions delivered by the manufacturer, however, with certain modifications; generally it uses genetically modified yeast cells of *Saccharomyces cerevisiae* with human estrogen hERα or androgen hAR receptors stably integrated into the main chromosome of the yeast cells. Yeasts exposed to compounds that have endocrine potential produce β-galactosidase, which oxidizes the CPRG dye in growth medium. The interpretation occurs by measuring the density of the cell suspension and the color saturation of the oxidized dye. Furthermore, the cells also contain an expression plasmid carrying the lacZ reporter gene encoding the enzyme β-galactosidase and means responsive to estrogens (YES) or androgen (YAS). The yeast cells were cultured from the filter papers in growth medium (basic medium with a vitamin solution, solution of L-threonine, L-aspartic acid and copper (II) sulfate (VI)). 5 mL of growth medium was transferred to a labeled culture bottles with caps with a gas permeable filter, afterwards the yeast disks were sterilely transferred and placed on an orbital shaker set at 32 °C temperature and 100 rpm for 48 hours. 100 µL of DMSO was added to each control vial containing standards: E2 (control of YES agonist), DHT (control of YAS agonist), HT (control of YES antagonist), FL (control of YAS antagonist). Test plates were prepared in such a way that the controls were in duplicate in eight serial dilutions. Final hormone concentration in positive controls ranged from 0.10 to 100 µM. 80 µL of 6 mM CRPG dye was added to each assay well. Next, 100 µL of YES and YAS suspension of yeast culture was added into agonist and antagonist YES and YAS plates, respectively. Assay plates were sealed with semi-permeable membranes and placed in the bag zipper moistened with watered gauze on an orbital shaker for 48 h at 32 °C 100 rpm. After 48 h of incubation, a cell density (by OD) was read at a wavelength of 690 nm and color intensity at a wavelength of 570 nm was determined. For the oestrogenic activity, the growth factor (G) and induction ratio (I_R) according to the equations:

$$G = \frac{A_{690,S}}{A_{690,N}} \quad (1)$$

$$I_R = \frac{1}{G} \cdot \frac{(A_{570,S} - A_{690,S})}{(A_{570,N} - A_{690,S})} \quad (2)$$

where $A_{690,\,S}$ and $A_{570,\,S}$ is absorbance of samples respectively at 690 nm and 570 nm and $A_{690,\,N}$ and $A_{570,\,N}$ is absorbance of the solvent control respectively at 690 nm and 570 nm. The determinations were repeated three times.

For the data assessment, the criterion was adopted that the tested sample has agonistic YES/YAS properties if the value of the induction coefficient ≥1.5 (for control solutions) and shows antagonistic YES/YAS properties if the value of the induction factor ≤66.7 % of the value obtained for the control sample.

2.5. FTIR studies

The FTIR spectra of crystalline products were recorded using Nicolet iS50 FT-IR spectrometer equipped with the Specac Quest single-reflection diamond attenuated total reflectance (ATR) accessory. Spectral analysis was controlled by the OMNIC software package. Polymers were identified by comparison with the reference spectra of the commercially available infrared library database implemented in OMNIC: HR Nicolet Sampler Library.

2.6. Chemometric studies

2.6.1. Experimental Design and Modeling

To assess the impact of various experimental conditions (resembling actual conditions to which different packaging materials are exposed), such as temperature, extraction time, and the concentration affecting the ecotoxicological response, an experimental design approach was used. A full factorial experimental design with two levels of variation of the input factor (2^n type) was chosen. The input factors were temperature (X_1), extraction time (X_2) and concentration (X_3). The output function was the ecotoxicity assessment value determined by the two major ecotoxicity tests: Microtox and XenoScreen (with its four options: YES+, YES-, YAS+ and YAS-). Thus, for each experiment, five output values were recorded (Brereton, 2007). The scheme of the experimental design is presented below in Table 2.

Table 2. Experimental design 2^3

Exp. No.	X_0	X_1	X_2	X_3	X_{12}	X_{13}	X_{23}	X_{123}	Y_{1-5}
1	+1	+1	+1	+1	+1	+1	+1	+1	
2	+1	-1	+1	+1	-1	-1	+1	-1	
3	+1	+1	-1	+1	-1	+1	-1	-1	
4	+1	-1	-1	+1	+1	-1	-1	+1	
5	+1	+1	+1	+1	+1	-1	-1	-1	
6	+1	-1	+1	-1	-1	+1	-1	+1	
7	+1	+1	-1	-1	-1	-1	+1	+1	
8	+1	-1	-1	-1	+1	+1	+1	-1	

For each of the input factors, two levels of variation were chosen, coded as +1 (high level) and -1 (low level). For the temperature, these levels were 121°C and room temperature; for time, they were 336 and 48 hours; and for concentration, they were the highest and lowest concentrations studied from the experimental scheme. Some minor variations were necessary for cups as the subject (no saliva as extraction media; only two factors were varied for DMSO) of the design, but the general principle was maintained. The experimental design was applied to each type of packaging material (cans, multilayered composite packaging and cups) and for each of the extraction media (water, ethanol, acetic acid, DMSO and saliva).

The experimental design allows for the creation of a polynomial model of the system studied of the form $Y = a_0 + \sum a_i X_i + \sum a_{ij} X_i X_j + a_{ijk} X_i X_j X_k$. The model reveals the impact of each input factor on the values of the output function and, additionally, the options for mixed interaction between the inputs leading to changes in the output. The sign and the values of each regression coefficient are measures of the weight and the direction of effect of the input factors. Using the experimental design above, eight regression coefficients were calculated:

a_0: intercept; assessment of the "conditional average" value of the output for the experimental conditions;
a_1–a_3: coefficients assessing the individual (single) impact of each of the factors studied on the output function;
a_{12}, a_{13}, and a_{23}: coefficients assessing the mixed (by couples) impact of the combination of each pair of factors studied on the output function; and
a_{123}: coefficient coefficients assessing the mixed (triple) impact of the combination of all three factors studied on the output function.
The model is tested for homogeneity of variance, significance of the regression coefficients and validity.

Additionally, hierarchical cluster analysis (HCA) and principal component analysis (PCA) were applied for data interpretation. For each type of packaging material, a data set was organized having dimensions of [25x8] for cans and multilayered composite packaging and [15x8] for cups. As objects, the packaging material in different extraction media were included, and the features (variables) describing the objects were the regression coefficients calculated by the experimental design procedure (from a_0 to a_{123}). It must be mentioned that the cup packaging material set has a lower dimension since only three solvents were included as extraction media (water, ethanol and acetic acid). The major goal of the chemometric data mining was to reveal specific patterns of similarity between the objects and between the variables. This could explain in a proper way any hidden relationships between the effects of the experimental conditions affecting the toxicity records for different packaging materials. Both multivariate statistical methods are well documented and do not need a detailed description (Masserat and Kaufman, 1987). The hierarchical cluster analysis was performed on standardized input data (z-standardization) using Ward's method of linkage and the squared Euclidean distances as measures of similarity. The cluster significance was determined by the criterion of Sneath. Additionally, PCA was performed (Varimax rotation mode) to confirm the results of clustering.

2.6.2. MANOVA studies

Multivariate analysis of variance (MANOVA) was used to evaluate the effects of the three main factors of solvent, temperature (temperature regime for the cups), and contact time in addition to solvent interactions with the other factors on the respective dependent variable (acute toxicity or endocrine-disruption potential). For the can and multilayer composite packaging experiments, three different food simulants (distilled water, ethanol, and acetic acid) were used since in the cup experimental design DMSO and saliva were also included. The three-way MANOVA procedure compares the acute toxicity and endocrine-disruption potential results obtained under different experimental conditions using different food simulants. For each type of packaging, three independent variables (factors) were considered in the factorial design: simulant, temperature (temperature regime for cup) and contact time. For can and multilayer composite packaging lining extracts, each of the factors of temperature and contact time takes three levels. This leads to 9 experiments for each food simulant, as conditions for each experiment are obtained by combinations of temperature and contact time levels (refer to Supplementary Table 1.). For cup extracts, the contact time takes five levels, which were performed at three temperature regimes: at 25°C, after a microwave oven heating procedure and after using hot solvent (the data are presented in Supplementary Table 1) for each food simulant. Each experiment from both experimental designs was run in triplicate for acute toxicity and in duplicate for the determination of endocrine-disruption potential.

3. Results and discussion

In Supplementary Table 2., the coefficients of all model parameters calculated for all types of packaging and extraction media are given. For convenience, the coded names of the objects are given as follows—the first two letters are for the type of packaging, the next letters indicate the solvent medium, and the last two indicate the ecotoxicity test applied (e.g., Can-Water_MT means cans in water medium tested by Microtox).

3.1. Discussion on experimental design results

3.1.1. Extraction medium and package type assessment with respect to toxicity tests

a) Microtox test results

It can be noticed (refer to Supplementary Table 2.) that the ethanol solution shows similar behavior when tested in the cases of all the packaging; the same holds true for acetic acid, where the highest toxicity values are recorded. Both cases for saliva are also identical for the can and multilayered composite packaging experiments. Surprisingly, MT toxicity records in water medium are very high for cans in contrast to the very low levels for multilayered composite packaging and cup extracts. DMSO medium is also highly toxic for cans and much lower for multilayered composite packaging.

b) XenoScreen test results

The same assessment was done for all four XenoScreen tests (see Supplementary Table 3.), YES+, YES-, YAS+ and YAS-, for all tested media and types of packaging. Again, a_0 values were used for the comments and conclusions. As shown in Supplementary Table 3., YES+ and YAS+ tests are very similar in that they indicate low activity for all media and all types of packaging. The records for YES- are highest for all cases with a maximum for cans. YAS- records resemble those for YES- with non-significant differences for the different extraction media.

3.1.2. Experimental conditions impact

The assessment is based on the values of the regression coefficients a_1–a_3, which indicate the weights of temperature, time of extraction and concentration on the output function records (toxicity). The temperature impact for Microtox testing (Supplementary Table 4.) is dominantly positive (a temperature increase leads to an increase in toxicity) for cans (DMSO and saliva media), for multilayered composite packaging and for cups in ethanol medium. A significant negative temperature impact is observed for multilayered composite packaging and cups in water medium. All other temperature effects are negligible. The time impact for Microtox testing (Supplementary Table 4.) is dominantly negative (a decrease in time corresponds to an increase in toxicity) except for two cases: multilayered composite packaging and cup packages in ethanol solution. The concentration impact for Microtox testing (Supplementary Table 4.) is dominantly positive with a few non-significant exceptions (the concentration increase causes toxicity levels to increase). In general, the regression coefficients indicating the impact of the mixed interactions in the system (temperature/time, temperature/concentration, time/concentration and temperature/time/concentration) are statistically non-significant and do not contribute to the interpretation of the model. The impacts of the experimental factors on the toxicity records by all the XenoScreen tests are much lower and insignificant compared with those indicated by Microtox. In Table 3, a short summary is given of the impact shown by a_1, a_2 and a_3 in all cases tested by XenoScreen.

Table 3. Summary of the experimental factors impact on toxicity indicated by XenoScreen tests (only significant effects are presented)

	a_1	a_2	a_3
YES +	Non-significant impact	Non-significant impact	Non-significant impact
YES-	***Cans – acetic acid*** ***Cans – saliva (positive impact)***	***Cups – acetic acid (negative impact)*** ***multilayer composite packaging - DMSO (positive impact)***	***Cans – water*** ***Cans – ethanol*** ***Cans – acetic acid*** ***Cans – DMSO (positive impact)***
YAS+	Non-significant impact	Non-significant impact	Non-significant impact
YAS-	Non-significant impact	***multilayer composite packaging - saliva (negative impact)***	Non-significant impact

3.2. MANOVA results and discussion

3.2.1. Microtox

a) Cans

The MANOVA model for the evaluation of the acute toxicity data for the can lining extracts exhibits a significant influence by all the main effects (solvent, temperature, and contact time) and their interactions on acute toxicity (Supplementary Fig. 1. and Fig. 2.). The most toxic are the acetic acid extracts with a mean predicted bioinhibition value of 93.82%, followed by water, ethanol and DMSO extracts with bioinhibition values in the range 65-72% (Supplementary Fig. 1a). The extracts of the last solvent, saliva, are not toxic, since they are accompanied by an absence of bioluminescence inhibition. Generally, for the other two factors of temperature and contact time, acute toxicity significantly increases with an increase in each independent variable (Supplementary Figs. 1b and 1c). Such an increase is more pronounced for temperature, while for contact time, the maximum bioinhibition is at 48 hours.

The solvent interaction effect plots with contact time and temperature for can lining extracts are presented in Fig. 2. The solvent-contact time interaction plot (Fig. 2a) exhibits two groups of solvents. The first group of solvents consisting of water, ethanol, acetic acid and DMSO has minimum extract acute-toxicity levels at 12 h and maximum levels at 48 h following contact time, effecting the shape presented in Fig. 2b. The toxicity of saliva extracts decreases with an increase in contact time, and the negative bioinhibition value at 336 h (increase in bioluminescence) is an indication that hormesis occurs under the conditions of the longest contact times. The solvent-temperature interaction shows a quite different behavior for the solvents used for can-lining extracts (Fig. 2b). Water, acetic acid and saliva extracts have the lowest acute toxicity levels at 25°C, and their toxicity increases with increasing temperature as the effect is more pronounced for the water and saliva solvents. The acute toxicity of ethanol and DMSO extracts does not possess a clear relationship with temperature, and it is worth mentioning that the highest bioinhibition values of DMSO extracts were obtained at 25°C.

Fig. 2.

b) Multilayer composite packaging

The MANOVA model of the acute toxicity data for the multilayer composite packaging lining extracts shows the significance of all main effects and their interactions on the acute toxicity of the extracts. Again, the most toxic are the acetic acid extracts with bioinhibition values for all obtained extracts of 100% (Supplementary Fig. 2a). Similarly, in the can-lining extract experiment, acetic acid is followed by less toxic water, ethanol and DMSO extracts. The toxicity of the multilayer composite packaging extracts of these solvents (bioinhibition values between 20 and 45%) is significantly lower than that of the can-lining extracts. Following this trend, the multilayer composite packaging saliva extracts are characterized by an increase in bioluminescence with a mean predicted bioinhibition value of -28.76%. The temperature and contact time do not strongly affect the solvent extract acute toxicity (Supplementary Fig. 2b and 2c), which is an indication that migration of toxic compounds occurs dominantly at the lowest levels of both factors, namely, 12 h and 25°C.

The solvent-contact time and solvent-temperature interaction plots confirm the small influence of extraction time and temperature on the acute toxicity of particular solvent extracts (Figs. 3a and 3b). An exception could be noted (Fig. 3a) in the solvent-contact-time plot that showed more pronounced hormesis of saliva extracts at 336 h. The solvent-temperature interaction plot (Fig. 3b) shows the similar behavior of DMSO and saliva with the can and multilayer composite packaging extracts, with maximum bioinhibition values for DMSO and saliva extracts at 25°C and 121°C respectively. In contrast to the can-lining experiment, the maximal acute toxicity of acetic acid multilayer composite packaging extracts is at 121°C, which is an indication for additional migration of toxic compounds at the highest temperature level.

Fig. 3.

c) Cups

The factors studied (and their interactions) have a significant effect on the acute toxicity of the extracts of the internal surfaces of cups. The solvent extract toxicity increases in the order of water, ethanol, and acetic acid with bioinhibition values similar to those of multilayer composite packaging lining extracts (Supplementary Fig. 3a). The increase in contact time is accompanied first by a decrease in toxicity and achieving the maximum bioinhibition values at prolonged extraction times (Supplementary Fig. 3b). Such dependence could be explained by transformations of more toxic substances to less toxic products during the extractions with durations of 1 to 2 hours, followed by migration of toxic compounds during prolonged extractions. Different temperature regimes do not strongly affect the acute toxicity of solvent extracts, as the most toxic are extracts obtained after hot solvent treatment (Supplementary Fig. 3c.). It should be mentioned that during microwave-assisted extraction, the heat and mass gradients work in one and the same direction, contrary to the other temperature regimes.

The solvent-contact time interaction plot (Fig. 4.) shows the different behaviors of the solvent extracts. The acute toxicity of the ethanol extracts increases with increasing extraction time, since the toxicity of the aqueous extracts decreases. The acetic acid extracts follow the pattern presented in the plot of the contact-time effect (Fig. 4a). The solvent-temperature regime interaction plot (Fig. 4b) confirms the small effect of temperature regimes on the acute toxicity of extracts, as the only exception is the low bioinhibition value of ethanol extracts obtained after microwave treatment.

Fig. 4.

3.2.2. XenoScreen YES/YAS

a) Cans

The MANOVA implementation to determine the endocrine-disruption potential of the can-lining extracts exhibits a clear difference between their estrogenic and androgenic disruption potential. The extracts of all solvents possess higher androgenic disruption potential than estrogenic disruption potential (Fig. 5.). Significant androgenic agonistic potential (ratio higher than 1 with respect to control values) has acetic acid extracts followed by DMSO and ethanol extracts, since water extracts possess significant androgenic antagonistic potential. The time-effect plot presented in Fig. 6. confirms the difference between their estrogenic and androgenic disruption potential. Similar to the acute-toxicity study of can-lining extracts, the time-effect plot notes the maximum androgenic disruption potential values at 48 h.

Fig. 5.

Fig. 6.

The temperature does not strongly affect the solvent extract endocrine-disruption potential (Fig. 7.), as this effect is not significant for estrogenic and androgenic antagonistic potentials. It seems that for both can studies related to acute toxicity and endocrine-disruption potential, the migration of toxic compounds occurs predominantly at 25°C.

Fig. 7.

The solvent-contact time interaction plot (Fig. 8.) shows that the highest androgenic disruption potential possessed by the can-lining extracts is obtained at 48 h. It should be mentioned that increasing contact time leads to an increase in androgenic disruption potential of the water extracts, which could be caused by the additional migration of toxic compounds during longer extraction procedures.

Fig. 8.

In general, the solvent-temperature interaction plot (Fig. 9.) confirms the small effect of temperature on the endocrine-disruption potential of the extracts. As exceptions, the increase in the androgenic disruption potential of water extracts and the decrease in androgenic antagonistic activity of ethanol extracts could be mentioned. The water extracts exhibit the same behavior as at prolonged extractions, which proves that migration of compounds with higher androgenic disruption potential occurs at higher temperatures and contact times. The decrease in the androgenic antagonistic activity of ethanol extracts with increasing temperature could be explained by the transformation of migrating substances to products with lower androgenic disruption potential.

Fig. 9.

b) Multilayer composite packaging (Supplement)

The MANOVA model for the endocrine-disruption potential of multilayer composite packaging lining extracts shows that temperature has no significant influence on the estrogenic disruption potential of extracts, and the same holds true for contact time regarding their androgenic agonistic activity. The solvent effect plot (Supplementary Fig. 4.) for multilayer composite packaging lining extracts shows the difference between their agonistic and antagonistic disruption potentials. Extracts of all solvents possess significantly higher estrogenic and androgenic antagonistic activity than agonistic ones. The acetic acid (YAS-), DMSO (YES-) and saliva (YES-) extracts show significant endocrine-disruption potential.

The time-effect plot does not reveal any large influence of contact time on the endocrine-disruption potential of the extracts model studied (Supplementary Fig. 5.). Only the increasing of androgenic antagonistic activity with an increase in contact time could be excluded from this tendency.

Taking into account the MANOVA results, the influence of temperature on the endocrine-disruption potentials of extracts could be discussed only for their androgenic disruption potential. The impact of temperature on androgenic agonistic activity of extracts is not well outlined, but extracts obtained at 65°C show a well-pronounced androgenic antagonistic maximum (Supplementary Fig. 6.). It could be concluded that migration of compounds with high androgenic antagonistic activity occur at 65°C and 336 h contact time.

The solvent-time interaction plot (Supplementary Fig. 7.) confirms significant endocrine-disruption potential of acetic acid (YAS-), DMSO (YES-) and saliva (YES-, YAS-) extracts. The highest androgenic antagonistic activity of acetic acid extracts obtained after 336 h supports the previously mentioned increase with time. The solvent-temperature interaction plot shows that the maximum values of antagonistic disruption potential for the acetic acid, DMSO and saliva extracts are obtained at 65°C (Supplementary Fig. 8.). It should be mentioned that both interaction plots show significant estrogen and androgen antagonistic potential in the saliva extracts.

3.3. Multivariate statistics

In the figures below (Supplementary Figs. 9-11), the graphical output of the cluster and principal components analysis is shown. It must be kept in mind that the chemometric analysis aims to assess the effect of the experimental conditions on the toxicity record of three different types of packaging material. This assessment is based on the results of the polynomial model obtained by the experimental design procedure. The important features are therefore the regression coefficient showing the effects of various experimental conditions in different extraction media and for different packaging materials.

a) Cans

In Supplementary Fig. 9a-c, the results from the hierarchical clustering of the variables (regression weights) and of the various toxicity tests applied to the packaging materials for cans in different extraction media are shown. Additionally, the biplot of PCA for the relationship between the factor loadings for two identified latent factors is presented. The same scheme is used for the other two packaging materials – multilayered composite packaging and cups.

Based on data presented in Supplementary Fig. 9a, it is evident that a close relationship exists between the features assessing the “average” toxicity (a_0), the temperature effect (a_1) and the concentration effect (a_3) for can packaging. The effect of the time of extraction (a_2) is not directly related to the other impacts and influences of mixed effects with time involvement. It could be concluded that the toxicity of the can extracts is subject to two major impacts—one related to concentration and temperature, and the other, to the time of extraction. This pattern separation has to be considered in relationship with the results shown in Supplementary Fig. 9b. All XenoScreen toxicity tests show a high level of similarity and form a very homogeneous cluster. This might mean that the effect of the extraction media is negligible. On the other hand, the Microtox test indicates variability with respect to the extraction media: Saliva and ethanol are outliers showing some resemblance to the XenoScreen tests, and water and DMSO are relatively similar to each other but very different from the XenoScreen tests. In Supplementary Fig. 9c, the relationship previously mentioned for the linkage between the regression coefficients as indicators for experimental factors impact is proven: a_0, a_1 and a_3 belong to one latent factor, and a_2 belongs to another.

b) Multilayer composite packaging

As seen in Supplementary Fig. 10a, the clustering of the regression coefficients for multilayer composite packaging is slightly different from that of the cans. Here, the “average” toxicity weight a_0 is related to a_2 and a_3 (the time of extraction and concentration), and a_1 (temperature impact) differs from the rest. This might mean that for multilayer composite packaging, the temperature impact is more specific, as the time of extraction impact was for can packaging. This is entirely confirmed by the biplot of factor loadings (Supplementary Fig. 10c) from PCA. The linkage between the different objects of multilayer composite packaging tested for toxicity (Microtox and XenoScreen) in different extraction media is the same as in the case of can packaging: non-specific and homogeneous (with respect to extraction media) results with XenoScreen and more specific with Microtox. Again, testing the toxicity using Microtox indicates significant differences for water, ethanol and saliva media and a slight resemblance between acetic acid and DMSO media with XenoScreen testing.

c) Cups

The results for the cup packaging material are obtained from the results of fewer experiments as already indicated. In Supplementary Fig. 11a, the clustering of a_0 and a_3 is obvious, since a_1 and a_2 are joined together. This means that the concentration factor is significant for the general toxicity, and the time and temperature factors form the other pattern of impact. The principal component analysis (biplot diagram in Supplementary Fig. 11c) confirms this conclusion. In Supplementary Fig. 11b, the non-specificity of the XenoScreen tests is indicated again. The Microtox test is seriously affected by the ethanol media (an outlier), and the other two media tested (water and acetic acid) are similar to each other but different from XenoScreen.

3.4. Results of FTIR studies

The spectra are presented in the electronic supplementary materials to this paper (Supplementary Figs. 16-18, respectively, for the spectra of can linings, multilayer composite packaging and cups). Each spectrum of the lining after extraction with a given solvent (for given time and temperature regime) was compared in reference to the spectrum of the “original” lining, i.e., before the extraction. The bands missing after the extraction are indicated with the blue arrows. The new bands that emerged or were definitely enlarged (as a result of the extraction) are denoted with the red arrows.

FT-IR ATR spectra of the can lining confirm the chemical character of the polymer as an epoxy resin mixture (Supplementary Fig. 12). They also exhibit a very high degree of resemblance to the FT-IR spectra of the diglycidyl ether of the bisphenol A mixture (Supplementary Fig. 13). The correlation coefficients between the measured and library spectra are 67.41% for the epoxy resin and 67.37% for the spectrum of the diglycidyl ether of the bisphenol A mixture. The spectral similarity was also inspected visually. The major differences, i.e., additional bands that are found in the 2700-2100 cm^{-1} region, are most likely caused by the contamination of the resin during the manufacturing process. This additional absorption may be produced by the remnants of a catalyst (most probably thiocyanate) in the polymer. Instrumental studies aiming for its identification are being continued. As observed in all extraction experiments, the impurities responsible for the appearance of the additional bands were repeatedly eluted with every solution applied to the extraction (Supplementary Figs. 16a-y). This means they are not incorporated into the polymer chain but are only weakly interacting with it. Moreover, it is very likely that these impurities are water-soluble. Such conclusions are confirmed by the fact that the correlation percentage between the spectrum of the epoxy resin and the studied can lining increased to nearly 71% after the extractions (Supplementary Fig. 16).

The multilayer composite packaging lining is a pure polyethylene (93.99% resemblance, Supplementary Fig. 14), which is basically not affected by the treatment with various solutions up to 65°C. At higher temperatures, the polyethylene is probably oxidized and/or absorbs the organic molecules of the solvent, which results in the appearance of additional bands that are ascribed to the absorption by carbonyl groups (please refer to Supplementary Fig. 17 for details). Similar to multilayer composite packaging the cup lining is polyethylene (93.91% match, Fig. 15). The correlations between the spectra of samples of cups treated with the studied liquids and the spectrum of polyethylene remain within a very narrow range of 93.64-94.18%. The results indicate that the interior of the cup is practically unaffected by treatments with the test solutions (please refer to Supplementary Fig. 18 for details).

4. Conclusions

The massive production and extensive usage of various packaging materials such as cups, cans, and composites (e.g., TetraPak) require a careful assessment of the impact of different storage and packaging conditions (temperature, disinfection, preserving, extracting and washing solvents) on the pollution and toxic effects of the packaging that becomes waste. Since the variety of temperature, type of solvent and concentration influences is quite broad, it seems reasonable to interpret the experimental results by chemometric means, including carefully designed experiments. An additional problem is the involvement of different ecotoxicity tests to assess the possible hazardous impact of the packaging wastes after usage or in the case of prolonged food-package contact under unfavorable conditions. Finally, an objective comparison of the chemical composition of the package lining before and after extraction procedures seems important in order to correctly assess the toxicity hazards.

FT-IR ATR spectroscopy is a useful, routine technique that allows to monitor the chemical changes of a polymer coating without any special pretreatment of the samples, provided they adhere to the refracting crystal (Tiefenhaller et al., 2017, Nikafshar et al., 2017, Yao et al., 2017). As studied by Tiefenhaller et al. (2017) the thermal stability of diglycidyl ether of the bisphenol A varies greatly with thermal regime applied and FTIR studies are indispensable to study processes occurring there and versatile transformation products are generated in the process and released to surrounding environment. Manguia-Lopez and Soto-Valez (2001) studied degradation and migration processes on tuna fish and pepper cans with chromatographic and FTIR methods. Enhanced migration of BPA and BADGE derivatives was stated in case of thermal treatment of objects (impact of time was not so significant). FCM and food itself processing certainly has also impact the package material degradation but also on degradation of (on the other hand favorable and desired) antioxidants; such non-intentional impact of treatment and processing was studied e.g. by Alin and Hakkarainen (2011) with gas chromatography methods or Gulmine et al. (2003) with FTIR spectroscopy. The microwave heating (in comparison to conventional heating) caused significantly higher specific migration of antioxidants studied. In other works, Atek and Belhaneche-Bensemra (2005) studied (with FTIR methods) impact of simulation media and time/temperature regime on stability of poly (vinyl chloride) as well as specific migration; the applicability of spectroscopic methods was also confirmed in field of mass transfer studies. For these, among other, reasons Nikafshar et al. (2017) started searching for renewable bio-based epoxy resin that could possibly be used as can lining material however with reduced toxicity. The vanillin-based product was confirmed to possess similar mechanical behavior like currently used analogs of diglycidyl ether of the bisphenol A.

Utilizing biological methods in FCM degradation and SM studies is less common than application of instrumental techniques known since mid XIX century. Most of these biological studies involved acute toxicity, endocrine potential, genotoxicity, cytotoxicity and mutagenicity determination. In the work of Mertl et al. (2014) migration of xenobiotics from carton and polypropylene packaging into food simulants was studied with CALUX assay; antagonistic effects were noted for foil and carton for milk product extract while no androgenic actions could be noted. Endocrine potential of recycled and virgin paper kitchen rolls was also studied by Vinggaard et al., (2000). In this case estrogenic response was observed mainly for ethanol extracts of recycled paper. Test with HELN, HG5LN cell lines was used by Chevolleau et al. (2016) to assess toxicity/endocrine potential of water stored in PET and glass bottles under different treatment processes, although the approach suggested did not allow to obtain any distinctive measures of endocrine potential. Such potential was found by Wagner and Oehlmann (2011) that performed similar studies however utilizing E-SCREEN assay; significant estrogenic effects in a human cancer cell line from 19.8 to 50.2% compared to 17-estradiol was confirmed. Galotto and Ulloa (2010) studied estrogenic activity of simulated liquids extracts of plastic packaging. Fatty simulation products appeared to have the highest estrogen activity for paper/aluminum/PE composites. Acute toxicity with bioluminescent bacteria of extracts of metal cans with polymeric linings was studied by Szczepańska et al. (2017) and acetic acid extracts appeared to be the most toxic to bioindicating organisms.

Certainly more studies are being performed in this area however their description falls out of scope of this paper. Still, it should be stressed that none of approaches utilized all three (instrumental, biological and chemometric) methods to study degradation phenomena of FCM. In the present studies, all of these aspects mentioned above were taken into account by the application of a full factorial experimental design followed by i) regression modeling of the experimental effects on the ecotoxicity readings, ii) ANOVA and MANOVA estimation of the experimental conditions as significant factors affecting the toxicity results and iii) IRFT analysis of the packaging. It is shown that the effects of temperature and extraction solvents could be quantitatively assessed with good correlation between different statistical estimates. Comparison of the results of instrumental and biological studies confirm the assumptions that bioassays can serve as a valuable and validated tool in assessing the stability and impact of packages on goods stored both for short and long periods of time. In some cases, the bioassays were proven to be more sensitive and reliable than IR studies, especially when studies deal with assessing the impact of acidic or alcohol-containing stimulants. In this study, it was confirmed that instrumental screening studies are not precise enough to assess the change in composition of FCM after some basic treatment processes, reflecting those processes that naturally occur during packaging and storage. On the other hand, bioassays may deliver suitable information when the results of extract screening of materials are referred to validated results for their respective lining material and chemometric tools are utilized to assess these relationships. Certainly, the method described, although proven to be reliable tool in FCM studies, has its limitations; just to mention: i) necessity to use non-toxic simulation agents not always reflecting already standardized instrumental methodologies, ii) need of performing studies with certifies organisms from different trophic levels, iii) lack of sufficient number of correlation studies to explain observed toxicological responses under different extraction conditions, iv) involving time-tested and sometimes highly qualified personnel to perform instrumental, biological and chemometric studies.

In any case, the performance of future work is indispensable to i) identify the chemicals responsible for observable toxicological levels (currently being performed in many scientific centers and laboratories including those of the authors), ii) assess the applicability of other bioassays as presented in the methodology of this research, iii) construct a battery of bioassays enabling exposure and degradation assessment for different types of packages and FCM, being at the same time complementary and universal for various treatment regimes, iv) propose international standards on the application of certified bioassays as recognized tools in the assessment of exposure to pollutants migrating from and created within FCMs, and v) comprehensively estimate (with classical and biological tools) the impact of packaging material wastes reaching different environmental niches after being used by households and industry, as they pose a serious source of small molecules and microplastics.

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Figures captions:

Fig. 1. Schematic presentation of research performed in the study

Fig. 2. Solvent interaction effect plots for acute toxicity determination of can lining extracts with: (a) contact time [h] and (b) temperature [°C]

Fig. 3. Solvent interaction effect plots for acute toxicity determination of multilayer composite packaging lining extracts with: (a) contact time [h] and (b) temperature [°C]

Fig. 4. Solvent interaction effect plots for acute toxicity determination of cup internal surface extracts with: (a) contact time [h] and (b) temperature regime

Fig. 5. Solvent effect plot for endocrine disruption potential determination of can lining extracts

Fig. 6. Time effect plot for endocrine disruption potential determination of can lining extracts

Fig. 7. Temperature effect plot for endocrine disruption potential determination of can lining extracts

Fig. 8. Solvent-time interaction plot for endocrine disruption potential determination of can lining extracts

Fig. 9. Solvent-temperature interaction plot for endocrine disruption potential determination of can lining extracts

Figure 1

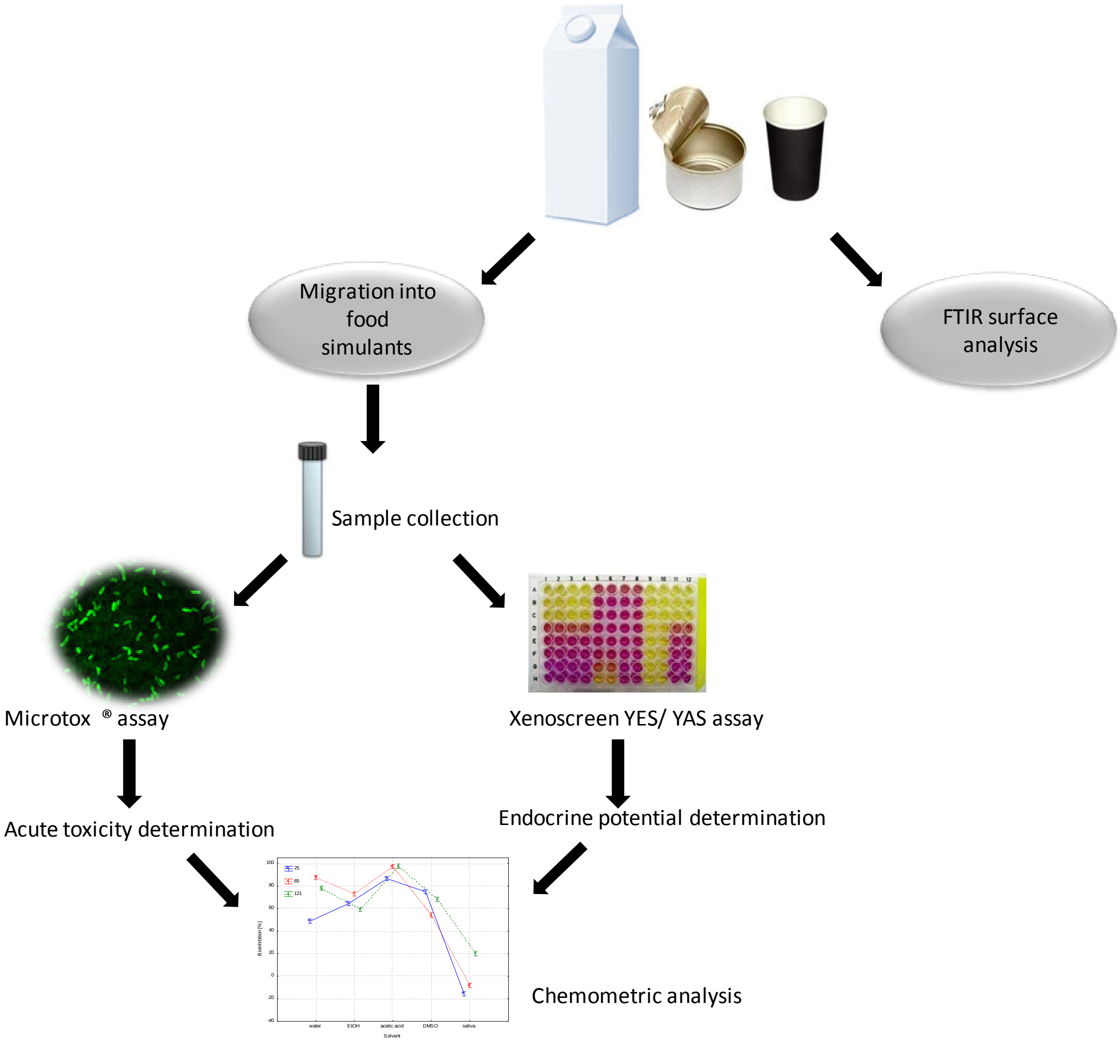

Fig. 1. Schematic presentation of research performed in the study

Figure 2

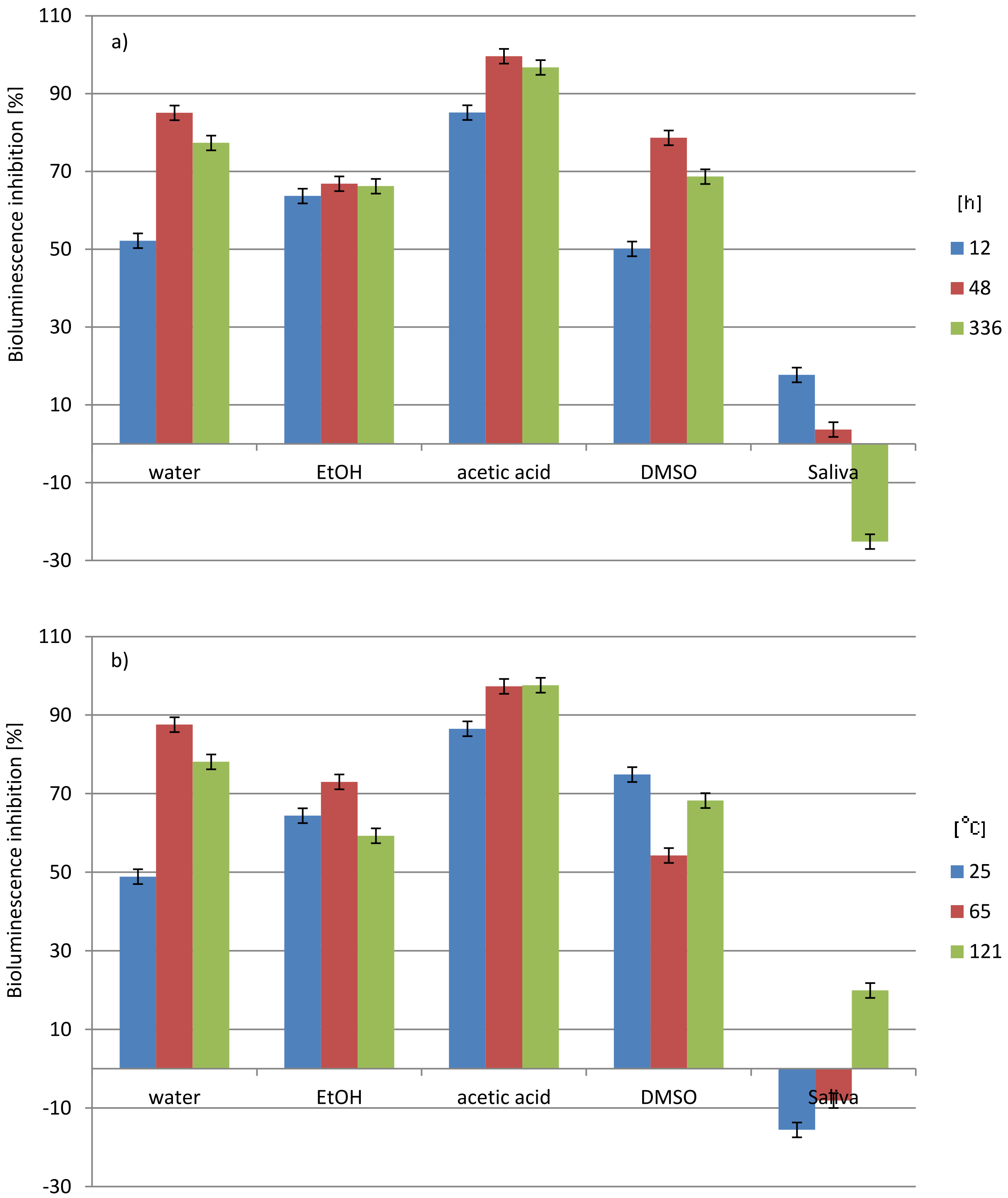

Fig. 2. Solvent interaction effect plots for acute toxicity determination of can lining extracts with: (a) contact time [h] and (b) temperature

Figure 3

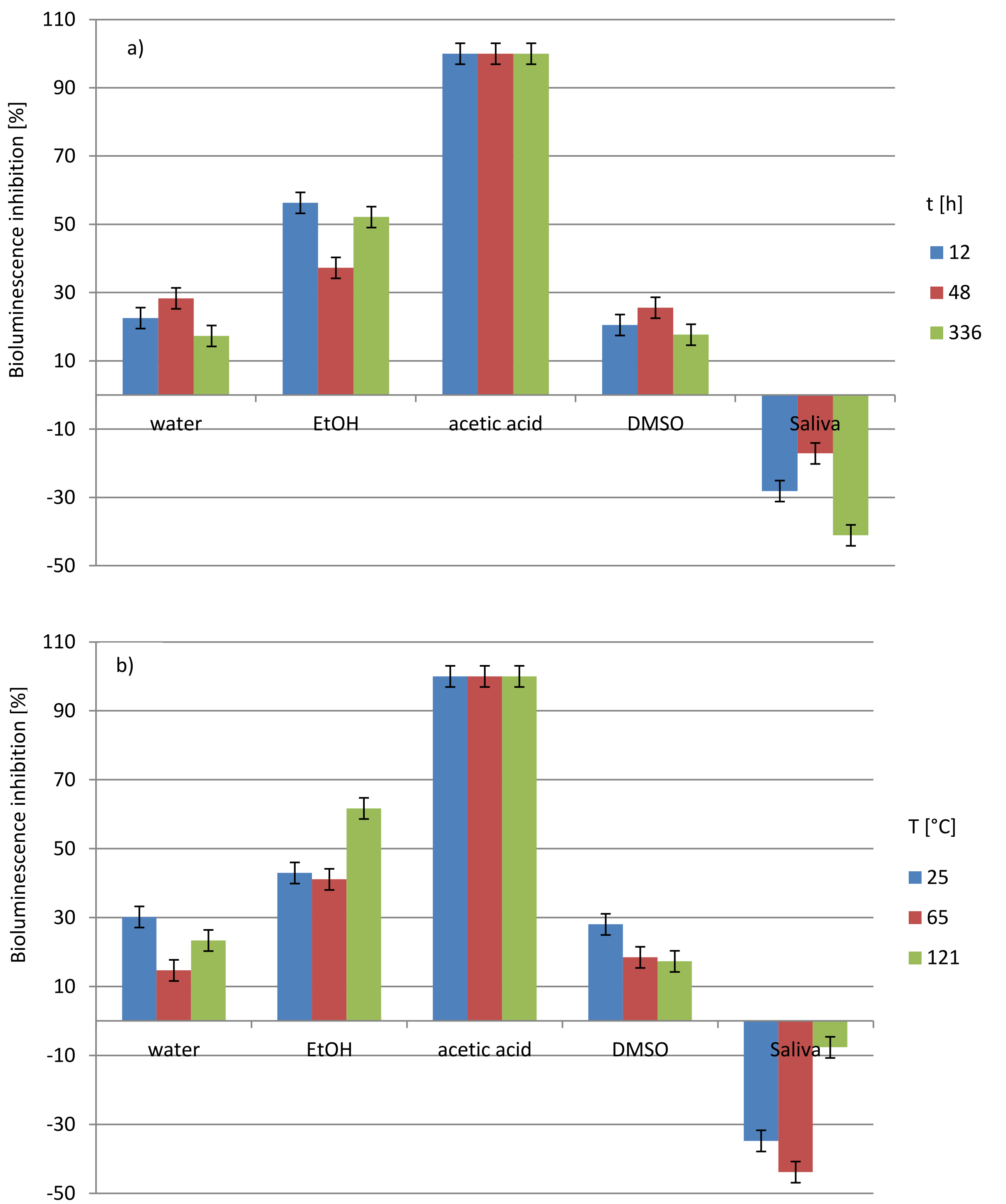

Fig. 3. Solvent interaction effect plots for acute toxicity determination of multilayer composite packaging lining extracts with: (a) contact time [h] and (b) temperature [°C]

Figure 4

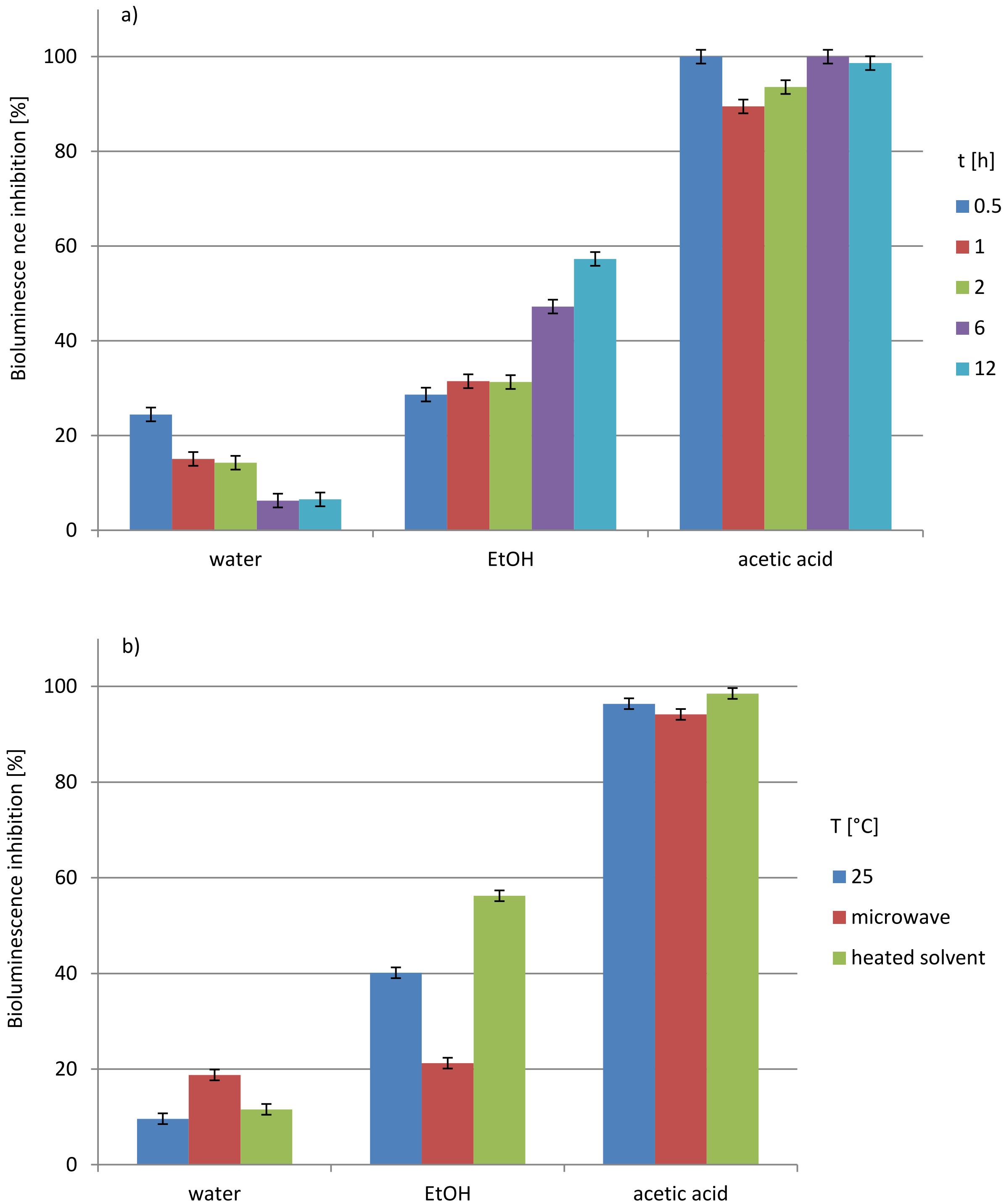

Fig. 4. Solvent interaction effect plots for acute toxicity determination of cup internal surface extracts with: (a) contact time [h] and (b) temperature regime

Figure 5

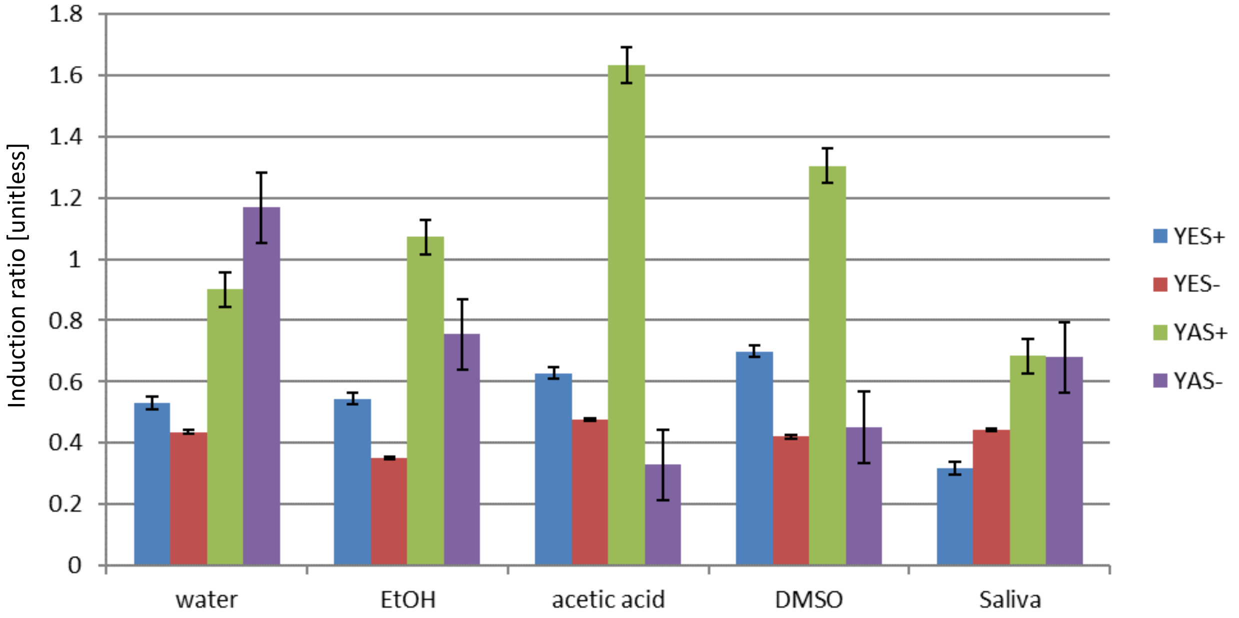

Fig. 5. Solvent effect plot for endocrine disruption potential determination of can lining extracts

Figure 6

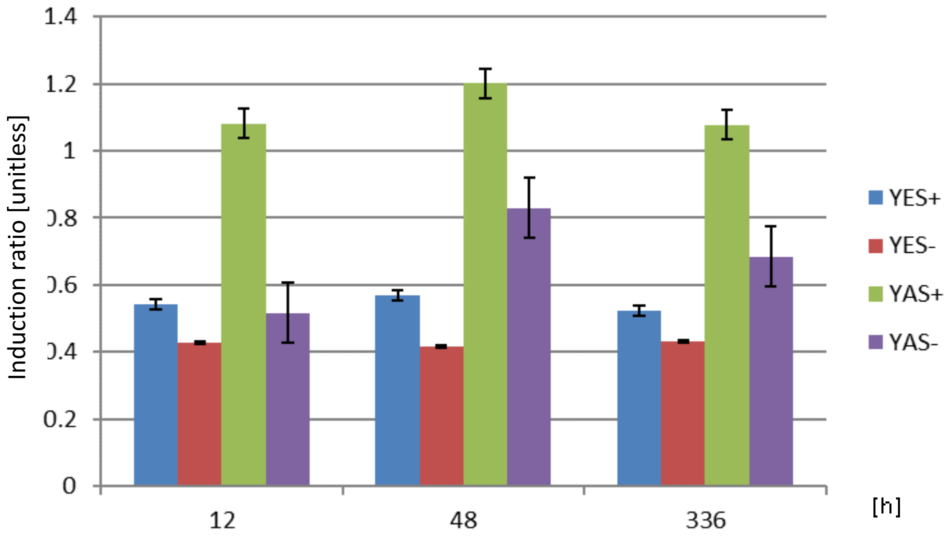

Fig. 6. Time effect plot for endocrine disruption potential determination of can lining extracts

Figure 7

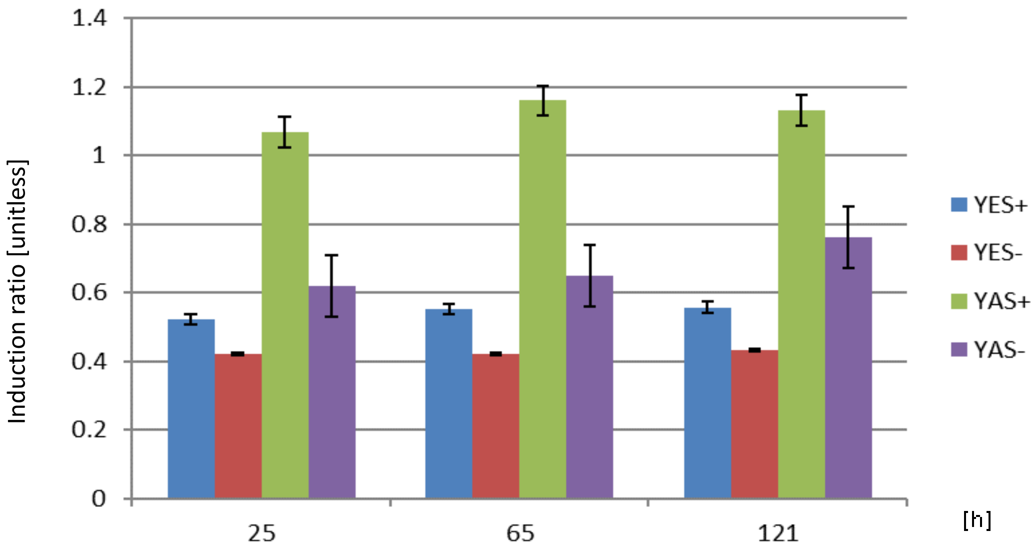

Fig. 7. Temperature effect plot for endocrine disruption potential determination of can lining extracts

Figure 8

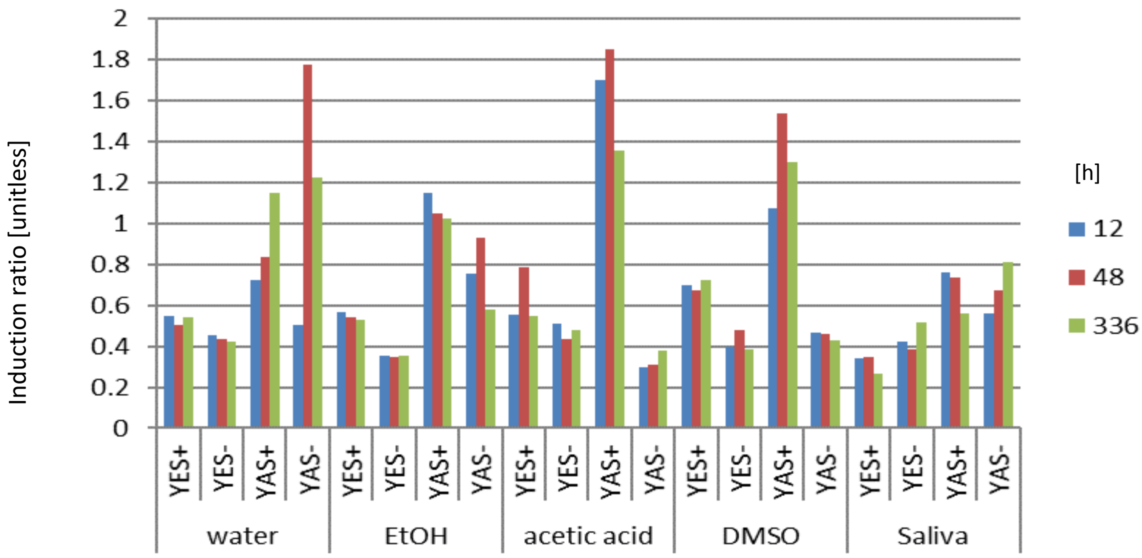

Fig. 8. Solvent-time interaction plot for endocrine disruption potential determination of can lining extracts

Figure 9

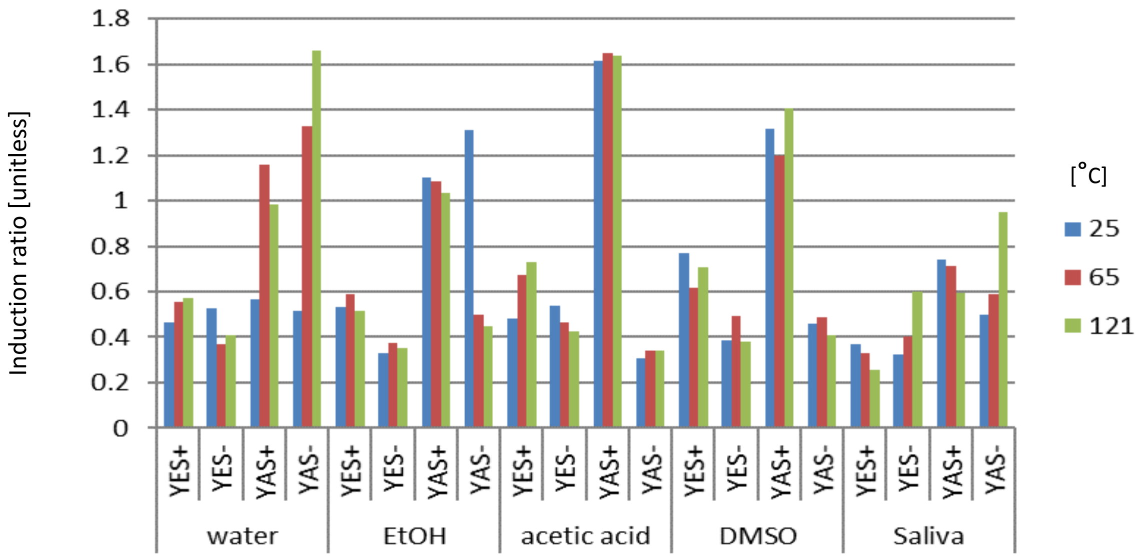

Fig. 9. Solvent-temperature interaction plot for endocrine disruption potential determination of can lining extracts