

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

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19
20 *Abstract:* The stability of the linings of packaging that is in contact with the goods stored has been of
21 major concern during decades of the development of packaging materials. In this work, an attempt
22 was undertaken to assess the applicability of using two bioassays (Microtox® and XenoScreen
23 YES/YAS) in estimating the stability of packaging (cans, caps, multilayer material) and the impact of
24 their degradation on the toxicity of some simulated media. The assessment of the impact of
25 packaging storage conditions (temperature, disinfection, preservation, extracting and washing
26 solvents) was planned and performed with i) regression modeling of the experimental effects on the
27 ecotoxicity readings, ii) ANOVA and MANOVA estimation of the experimental conditions as
28 significant factors affecting the toxicity results and iii) FTIR analysis of the packages. It is shown that
29 the effects of temperature and extraction solvents could be quantitatively assessed by the
30 agreement between all methods applied. It can be stated that temperature and acidity as well as the
31 alcohol content in the sensitive media have the greatest impact on the toxicity of the extract and
32 thus on the stability of the internal lining and the extractability of xenobiotics.

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

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40 *Highlights:*

- 41
42 - Simultaneous assessment of the impact of treatment conditions on packages ecotoxicity.
43 - Checking of the impact of temperature and extracting solvents on endocrine potential of packages.
44 - Comparison of the package lining spectra before and after extraction.
45 - Ecotoxicity parameters as a tool in packages lining stability assessment.

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36 1. Introduction

37 The introduction of packaging materials has revolutionized the sales of common products.
38 The use of packaging has mainly contributed to the extension of the expiration date of food products
39 and made their transport and storage easier. Currently, a vast majority of products are sold in such
40 packaging as plastics, paper, glass, metal and composite materials. Despite the clear benefits of these
41 materials, their long-term use has shown some negative aspects. Since the 1980s, we have known
42 that they might be an additional source of exposing people to xenobiotics (Lau and Wong, 2000). The
43 issue of food packaging safety is regulated by both the EU and domestic legislation. Nevertheless,
44 numerous studies have proven that the small-molecule components of packaging may be eluted
45 from the internal layer of a material due to the presence of the medium stored (Arvanitoyannis and
46 Kotsanopoulos, 2014; Ossberger, 2015). These facts have caused the issues related to the migration
47 of xenobiotics and factors that influence the intensification of the process to become one of the
48 leading topics of interest for researchers. Actions are being taken mainly to identify and
49 quantitatively determine the compounds released and eventually to assess the risk to humans
50 (Canellas et al., 2015; Guart et al., 2011). As can be easily concluded, this task is particularly difficult.
51 Due to a vast number of various types of substances used for the production of packaging materials,
52 there are hundreds of compounds that can migrate from food contact materials to food or food
53 simulated media additives starting from substances such as plasticizers, antioxidants, light and
54 thermal stabilizers, slip compounds, antistatic agents, lubricants, and monomers to heavy metals and
55 nanoparticles (García et al., 2006; Guart et al., 2011; Raptopoulou et al., 2014; Sanches-Silva et al.,
56 2009). It is obvious that the exposure to pollutants always has an unfavorable impact on an
57 organism. However, the threat is particularly severe in this case. A significant number of chemical
58 substances from the abovementioned groups (e.g., bisphenol A, bisphenol A diglycidyl ether and its
59 derivatives, phthalates, primary aromatic amines, perfluorinated compounds and some heavy
60 metals) are classified as endocrine-disrupting compounds (EDC) (Moreira et al., 2013; Moreta and
61 Tena, 2014; Pérez-Palacios et al., 2012; Pezo et al., 2012). The results of numerous studies indicate
62 that EDCs may alter the activity of natural steroid hormones by modifying their regulatory pathways,
63 interacting with steroid receptors and antagonizing endogenous hormones or simply mimicking
64 steroid hormone-dependent effects (Kudlak et al., 2015).

65 Although first mentions about the activity of these compounds appeared many years ago,
66 they are still the object of great interest for the researchers. More and more advanced techniques
67 used in the research provide novel information about harmful activity of these compounds. The
68 danger is much more significant than it was believed before. Therefore, it was necessary to
69 implement numerous amendments to the regulations concerning acceptable safe doses specified as
70 regards individual substances. The first regulatory standard for bisphenol A was established in 1988
71 and the oral reference dose was assessed on 50 mg/kg/day. Then the value was reduced to 4 mg/kg
72 of body mass (Ćwiek-Ludwicka, 2015). Regulations related to the acceptable limits of compounds
73 migrating from the surface of a package have also been tightened up. SML value for bisphenol A was
74 reduced in 2018 from 0.6 mg to 0.05 mg/kg of food or food simulant (Commission Regulation (EU)
75 2018/213). Supposedly, these values will be reduced again in the nearest future.

76 Additional problem connected with the phenomena of releasing compounds from the
77 surface of the package relies on the fact that apart from well-known starting substances, impurities
78 known as Non-Intentionally Added Substances (NIAS) can be present in food as well as their
79 transformation products. The difficulties related to the identification of all the released compounds
80 and the lack of knowledge about the toxicity of those compounds have resulted in an increase in the
81 importance of using bioanalytical techniques in such research areas (Maisanaba et al., 2014; Ozaki et
82 al., 2004; Wagner and Oehlmann, 2011). The main advantage of *in vitro* bioassays is the possibility of

83 specifying the actual influence of the overall migration, considering most of the interactions
1 84 occurring between pollutants released. This is particularly important in consideration of the fact that
2 85 we currently know that compounds coexisting in the mixture may interact and cause an increase
3 86 (synergism) or decrease (antagonism) in the final effect. There are many premises that such
4 87 interactions occur between compounds released from the surface of packaging materials (Hu et al.,
5 88 2014; Li et al., 2017; Wiczerzak et al., 2016). In our previous research, synergistic interactions were
6 89 found in mixtures that contain such compounds as BADGE·H₂O, BADGE·2H₂O or BFDGE (Szczepańska
7 90 et al., 2018).

11 91 Due to the properties of compounds released, researchers usually apply tests that enable
12 92 specifying the hormonal activity of the packaging samples. The XenoScreen YES/YAS, ERa and AR
13 93 CALUX served as analytical tools that enabled the confirmation that the compounds released from
14 94 some carton and polypropylene packaging show estrogenic activity (YES EEQ = 59.6±29.3 ng/L) (Mertl
15 95 et al., 2014). Another group of tests used in such studies includes those that provide information on
16 96 mutagenicity, genotoxicity and cytotoxicity of the given samples (i.e., extracts of the packaging).
17 97 Based on the results obtained with the use of the rec-assay and comet assay, it was possible to show
18 98 that the compounds released from the surface of paper and paperboard packaging cause weak
19 99 genotoxic and cytotoxic effect In the literature, there is some additional information on the
20 100 possibility of using bioassays in the research on paper and plastic packaging (Galotto and Ulloa,
21 101 2010; Ozaki et al., 2005). To the best of our knowledge, there is no information on the toxicity and
22 102 hormonal activity of compounds released from the internal surface of cans and multilayer composite
23 103 packaging. Thus, it also seems that the current research will be a significant contribution to present
24 104 knowledge and that it will be a perfect complement to the existing information on the influence of
25 105 compounds released from common packaging materials on living organisms. The main objective of
26 106 the research was the evaluation (with MANOVA data treatment) of the toxicity and endocrine
27 107 potential of the compounds released from packaging using the XenoScreen YES/YAS and Microtox®
28 108 biological tests.

33 109 2. Methodology

34 110 2.1. Experimental

35 111 Because one of the most commonly used types of food packaging is currently metallic and
36 112 multi-material composite packaging, cans, multilayered composite packaging and cups were chosen
37 113 for the study. The subjects of the study were 60 metal cans devoted to fish storage (total volume ca.
38 114 0.15 dm³), 56 paper hot-drink cups (0.20 dm³ volume) and 60 multilayered composite packaging units
39 115 commonly used for milk or juice storage (1 dm³ volume). None of the samples was in contact with
40 116 food prior to testing, and care was taken to avoid any contamination of the samples during sampling,
41 117 storage or transport. Two approaches were used in the study. The goal of the first approach was to
42 118 estimate the toxicity and endocrine potential of compounds released into simulant liquids, whereas
43 119 the second focused on determining whether and to what extent the exposure to the extraction
44 120 agents/conditions results in the degradation of the polymer layer. Fig. 1 shows the schematic
45 121 approach applied in studies. None of the samples were in contact with food prior to testing, and care
46 122 was taken to avoid any contamination of the samples during sampling, storage or transport.

47 123
48 124 **Fig. 1.**

49 125 50 126 2.1. Chemicals and reagents

51 127 Chemicals that were used for preparing simulant media were obtained from the following
52 128 suppliers: sodium chloride (CAS no. 7440-23-5) (Sigma Aldrich, Germany), dipotassium phosphate
53 129 (CAS no. 7758-11-4) (Ciech S.A., Poland), calcium chloride (CAS no. 7440-70-2) (Eurochem BGD,

130 Poland), magnesium chloride (CAS no. 7786-30-3), potassium chloride (CAS no. 7440-09-7),
131 potassium carbonate (CAS no. 584-08-7), lactic acid (CAS no. 79-33-4), urea (CAS no. 57-13-6) (POCH
132 S.A., Poland), ammonium hydroxide (25 % w/w) (CAS no. 1336-21-6), acetic acid (AcOH, 35-38% w/w,
133 CAS no. 64-19-7) (Chempur, Poland), distilled water, EDC-Pak cartridge (Merck, Germany). Microtox[®]
134 kit (2% NaCl, lyophilized *Vibrio fischeri* bacteria, Microtox Diluent, Microtox Acute Reagent, Osmotic
135 Adjusting Solution (OAS), and Reconstitution Solution (RS) were purchased from ModernWater Ltd.
136 (GB). Ethanol (EtOH, CAS no. 64-17-5), dimethyl sulfoxide (DMSO, CAS no. 67-68-5) and Parafilm[®]
137 were purchased from Sigma-Aldrich (Germany). All reagents were of analytical grade or higher
138 (reagents used for microbiological purposes). Reagents used for XenoScreen YES/YAS were
139 purchased from Xenometrics G. A. (Switzerland). These were: vials containing hER α yeast cells (for
140 YES assay) and hAR yeast cells (for YAS assay) on a filter paper, basal medium, vitamin, L-aspartic acid
141 (CAS no. 56-84-8), L-threonine (CAS no. 72-19-85) and copper sulfate solutions (CAS no. 7758-98-7),
142 CPRG (chlorophenol red- β -D-galactopyranoside) (CAS no. 99792-79-9), vials with 17 β -estradiol (CAS
143 no. 50-28-2), 5 α -dihydrotestosterone (CAS no. 521-18-6), 4-hydroxytamoxifen (CAS no. 68392-35-8),
144 flutamide (CAS no. 13311-84-7), DMSO (dimethyl sulfoxide) (CAS no. 67-68-5). 96-well plates, gas-
145 permeable plate sealers, culture flasks with gas-permeable filter cap were purchased from GenoPlast
146 Biochemicals (Poland). All reagents were of analytical grade purity or better in case of reagents for
147 microbiological purposes. The instruments and equipment used during the study were: Microtox[®]
148 500 from Modern Water Ltd. (GB), electronic multi- and single-channel pipettes from Eppendorf
149 (Germany), NaOH (CAS no. 1310-73-2) and HCl (CAS no. 7647-01-0) (purchased from Avantor
150 Performance Materials S.A. (Poland)), CP411 pH-meter from Metron (Poland), heater of Thermicon
151 P[®] (type K1253S) Heraeus Instruments (Germany), microwave heating device (Samsung ME 733K
152 (maximum power 1150 W) and shaker type water bath 357 from Elpan Laboratory Instruments
153 (Poland).

2.2. Preparation of simulant liquids

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156 All simulant solutions were prepared using reagents of analytical grade purity. Artificial saliva
157 was prepared in accordance to the guidelines described in the DIN:53160-1:2010-10 standard (NaCl
158 0.53 g/dm³, KCl 0.33 g/dm³, CaCl₂·2H₂O 0.15 g/dm³, K₂HPO₄·3H₂O 0.76 g/dm³, MgCl₂·6H₂O 0.17
159 g/dm³, K₂CO₃ 0.53 g/dm³, and 1% HCl 0.75 g/dm³). The pH of the solution was adjusted using a 1%
160 NH₃ solution to a value of 6.8. To minimize the problem of the background EDC pollution, the Milli-Q
161 water was additionally purified by the use of an EDC-Pak cartridge (for removing
162 endocrine-disrupting compounds) at the stage of the preparation of simulation liquids. Additionally,
163 to reduce the risk of contamination of the glassware with organics, an additional step of heating
164 utensils at 450°C for at least 4 hours was applied (Fierens et al., 2012). Simulation liquids were stored
165 at +4 °C prior to performing the extraction process.

2.3. Sample collection

166
167
168 The migration tests were carried out in accordance with the test procedure indicated in the
169 Commission Regulation (EU) No. 10/2011 and PN-EN 1186-1:2005 standard. Considering the fact that
170 the tested packaging is intended for storing a very wide range of products (such as fruits and
171 vegetables, meat products and various beverages), the simulation liquids most commonly used in the
172 migration studies (distilled water, acetic acid solution and ethanol solution) were used in the study
173 (standard procedures, e.g., the EU legislation recommends using 10% ethanol; however, due to the
174 high alcohol content, an unintended elevated toxicity was observed and a lower ethanol content had
175 to be utilized). Moreover, a 5% dimethyl sulfoxide (DMSO) solution and artificial saliva solution were
176 used to check the extraction strength of other solvents (DMSO is considered as a factor reflecting the
177 lipophilic properties of ingredients present in the product stored). The tests were performed using
178 the filling method: the packages were filled with their respective simulation fluids up to 5 mm below

179 the upper edge. Since cans and multilayered composite packaging are intended for the long-term
 180 storage of food, they were held at 60°C for 10 days (240h) after filling,. Additionally, in order to
 181 determine the effect of temperature on the degree of release of xenobiotics, the different kinds of
 182 packaging were also exposed to temperatures of 65°C and 121°C and the tests were also performed
 183 for 336h (these results are presented in manuscript). A different procedure was used in the case of
 184 research performed with cups. These were filled with boiled solvents up to 5 mm below the upper
 185 edge. Additionally, in order to determine the effect of microwave heating on the migration of
 186 chemical compounds from the cups, a microwave treatment was carried out. The treatment was
 187 stopped when the temperature of the solvents reached 95–100°C. All objects were shaken with
 188 orbital movement at 100 revolutions per minute (rpm) throughout the time period. Table 1 provides
 189 more detailed information on the conditions and methods applied in the studies.

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

211 reduction of natural light output emitted by the bacteria is proportional to the degree of toxicity of a
212 given sample. pH was adjusted to fall within the 6.5-7.5 range with NaOH and HCl. Acute toxicity was
213 determined by standard protocol using the Microtox[®] Analyzer Model 500 and serial dilutions.
214 Lyophilized reagent with *Vibrio fischeri* bacteria was hydrated with 1 mL of RS and maintained at
215 5.5±1.0°C, subsequently 100 µL of 10-fold diluted reconstituted Microtox Reagent bacterial solution
216 and 2 mL of samples were added into the vials. To produce a suitable osmotic pressure (above 2 %) OAS
217 was added to the vial with the highest concentration and proper dilutions were prepared. For
218 quality assurance of proper test run the following parameters according to producers guidelines
219 were assumed: for Microtox[®] I₀ of bacterial suspension >70 U (chromium sulphate was used as a
220 positive control of bacterial stock suspension test run).

222 2.4.2. XenoScreen YES/YAS methodology

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

$$245 \quad G = \frac{A_{690,S}}{A_{690,N}} \quad (1)$$

$$246 \quad I_R = \frac{1}{G} \cdot \frac{(A_{570,S} - A_{690,S})}{(A_{570,N} - A_{690,S})} \quad (2)$$

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

250
251 For the data assessment, the criterion was adopted that the tested sample has agonistic
252 YES/YAS properties if the value of the induction coefficient ≥ 1.5 (for control solutions) and shows
253 antagonistic YES/YAS properties if the value of the induction factor ≤ 66.7 % of the value obtained for
254 the control sample.

256 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.

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Table 2. Experimental design 2^3

Exp. No.	X_0	X_1	X_2	X_3	X_{12}	X_{13}	X_{23}	X_{123}	Ψ_{15}^{75}
1	+1	+1	+1	+1	+1	+1	+1	+1	
2	+1	-1	+1	+1	-1	-1	+1	-1	276
3	+1	+1	-1	+1	-1	+1	-1	-1	
4	+1	-1	-1	+1	+1	-1	-1	+1	277
5	+1	+1	+1	+1	+1	-1	-1	-1	
6	+1	-1	+1	-1	-1	+1	-1	+1	278
7	+1	+1	-1	-1	-1	-1	+1	+1	
8	+1	-1	-1	-1	+1	+1	+1	-1	279

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:

295 a_0 : intercept; assessment of the “conditional average” value of the output for the experimental
1 296 conditions;

2 297 a_1 – a_3 : coefficients assessing the individual (single) impact of each of the factors studied on the output
3 298 function;

4 299 a_{12} , a_{13} , and a_{23} : coefficients assessing the mixed (by couples) impact of the combination of each pair
5 300 of factors studied on the output function; and

6 301 a_{123} : coefficient coefficients assessing the mixed (triple) impact of the combination of all three factors
7 302 studied on the output function.

8 303 The model is tested for homogeneity of variance, significance of the regression coefficients and
9 304 validity.

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11
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14 305 Additionally, hierarchical cluster analysis (HCA) and principal component analysis (PCA) were
15 306 applied for data interpretation. For each type of packaging material, a data set was organized having
16 307 dimensions of [25x8] for cans and multilayered composite packaging and [15x8] for cups. As objects,
17 308 the packaging material in different extraction media were included, and the features (variables)
18 309 describing the objects were the regression coefficients calculated by the experimental design
19 310 procedure (from a_0 to a_{123}). It must be mentioned that the cup packaging material set has a lower
20 311 dimension since only three solvents were included as extraction media (water, ethanol and acetic
21 312 acid). The major goal of the chemometric data mining was to reveal specific patterns of similarity
22 313 between the objects and between the variables. This could explain in a proper way any hidden
23 314 relationships between the effects of the experimental conditions affecting the toxicity records for
24 315 different packaging materials. Both multivariate statistical methods are well documented and do not
25 316 need a detailed description (Masserat and Kaufman, 1987). The hierarchical cluster analysis was
26 317 performed on standardized input data (z-standardization) using Ward’s method of linkage and the
27 318 squared Euclidean distances as measures of similarity. The cluster significance was determined by
28 319 the criterion of Sneath. Additionally, PCA was performed (Varimax rotation mode) to confirm the
29 320 results of clustering.

30 321 31 32 33 34 35 36 37 38 39 322 2.6.2. MANOVA studies

40
41 323 Multivariate analysis of variance (MANOVA) was used to evaluate the effects of the three
42 324 main factors of solvent, temperature (temperature regime for the cups), and contact time in addition
43 325 to solvent interactions with the other factors on the respective dependent variable (acute toxicity or
44 326 endocrine-disruption potential). For the can and multilayer composite packaging experiments, three
45 327 different food simulants (distilled water, ethanol, and acetic acid) were used since in the cup
46 328 experimental design DMSO and saliva were also included. The three-way MANOVA procedure
47 329 compares the acute toxicity and endocrine-disruption potential results obtained under different
48 330 experimental conditions using different food simulants. For each type of packaging, three
49 331 independent variables (factors) were considered in the factorial design: simulant, temperature
50 332 (temperature regime for cup) and contact time. For can and multilayer composite packaging lining
51 333 extracts, each of the factors of temperature and contact time takes three levels. This leads to 9
334 experiments for each food simulant, as conditions for each experiment are obtained by combinations
335 of temperature and contact time levels (refer to Supplementary Table 1.). For cup extracts, the
336 contact time takes five levels, which were performed at three temperature regimes: at 25°C, after a

337 microwave oven heating procedure and after using hot solvent (the data are presented in
1 338 Supplementary Table 1) for each food simulant. Each experiment from both experimental designs
2 339 was run in triplicate for acute toxicity and in duplicate for the determination of endocrine-disruption
3 340 potential.
4 341

7 342 3. Results and discussion

9 343 In Supplementary Table 2., the coefficients of all model parameters calculated for all types of
10 344 packaging and extraction media are given. For convenience, the coded names of the objects are
11 345 given as follows—the first two letters are for the type of packaging, the next letters indicate the
12 346 solvent medium, and the last two indicate the ecotoxicity test applied (e.g., Can-Water_MT means
13 347 cans in water medium tested by Microtox).
14 348

17 348 3.1. Discussion on experimental design results

19 349 3.1.1. Extraction medium and package type assessment with respect to toxicity tests

21 350 a) Microtox test results

23 351 It can be noticed (refer to Supplementary Table 2.) that the ethanol solution shows similar
24 352 behavior when tested in the cases of all the packaging; the same holds true for acetic acid, where the
25 353 highest toxicity values are recorded. Both cases for saliva are also identical for the can and
26 354 multilayered composite packaging experiments. Surprisingly, MT toxicity records in water medium
27 355 are very high for cans in contrast to the very low levels for multilayered composite packaging and cup
28 356 extracts. DMSO medium is also highly toxic for cans and much lower for multilayered composite
29 357 packaging.
30 358

33 358 b) XenoScreen test results

35 359 The same assessment was done for all four XenoScreen tests (see Supplementary Table 3.),
36 360 YES+, YES-, YAS+ and YAS-, for all tested media and types of packaging. Again, a_0 values were used for
37 361 the comments and conclusions. As shown in Supplementary Table 3., YES+ and YAS+ tests are very
38 362 similar in that they indicate low activity for all media and all types of packaging. The records for YES-
39 363 are highest for all cases with a maximum for cans. YAS- records resemble those for YES- with non-
40 364 significant differences for the different extraction media.
41 365

44 365 3.1.2. Experimental conditions impact

46 366 The assessment is based on the values of the regression coefficients a_1 – a_3 , which indicate the
47 367 weights of temperature, time of extraction and concentration on the output function records
48 368 (toxicity). The temperature impact for Microtox testing (Supplementary Table 4.) is dominantly
49 369 positive (a temperature increase leads to an increase in toxicity) for cans (DMSO and saliva media),
50 370 for multilayered composite packaging and for cups in ethanol medium. A significant negative
51 371 temperature impact is observed for multilayered composite packaging and cups in water medium. All
372 other temperature effects are negligible. The time impact for Microtox testing (Supplementary Table
373 4.) is dominantly negative (a decrease in time corresponds to an increase in toxicity) except for two
374 cases: multilayered composite packaging and cup packages in ethanol solution. The concentration
375 impact for Microtox testing (Supplementary Table 4.) is dominantly positive with a few non-
376 significant exceptions (the concentration increase causes toxicity levels to increase). In general, the

377 regression coefficients indicating the impact of the mixed interactions in the system
 1 378 (temperature/time, temperature/concentration, time/concentration and
 2 379 temperature/time/concentration) are statistically non-significant and do not contribute to the
 3 380 interpretation of the model. The impacts of the experimental factors on the toxicity records by all the
 4 381 XenoScreen tests are much lower and insignificant compared with those indicated by Microtox. In
 5 382 Table 3, a short summary is given of the impact shown by a_1 , a_2 and a_3 in all cases tested by
 6 383 XenoScreen.

10 Table 3. Summary of the experimental factors impact on toxicity indicated by XenoScreen tests (only
 11 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

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

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

410 temperature, and it is worth mentioning that the highest bioinhibition values of DMSO extracts were
411 obtained at 25°C.

412

413

Fig. 2.

414

415

b) Multilayer composite packaging

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

427 The solvent-contact time and solvent-temperature interaction plots confirm the small
428 influence of extraction time and temperature on the acute toxicity of particular solvent extracts (Figs.
429 3a and 3b). An exception could be noted (Fig. 3a) in the solvent-contact-time plot that showed more
430 pronounced hormesis of saliva extracts at 336 h. The solvent-temperature interaction plot (Fig. 3b)
431 shows the similar behavior of DMSO and saliva with the can and multilayer composite packaging
432 extracts, with maximum bioinhibition values for DMSO and saliva extracts at 25°C and 121°C
433 respectively. In contrast to the can-lining experiment, the maximal acute toxicity of acetic acid
434 multilayer composite packaging extracts is at 121°C, which is an indication for additional migration of
435 toxic compounds at the highest temperature level.

436

437

Fig. 3.

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c) Cups

440 The factors studied (and their interactions) have a significant effect on the acute toxicity of
441 the extracts of the internal surfaces of cups. The solvent extract toxicity increases in the order of
442 water, ethanol, and acetic acid with bioinhibition values similar to those of multilayer composite
443 packaging lining extracts (Supplementary Fig. 3a). The increase in contact time is accompanied first
444 by a decrease in toxicity and achieving the maximum bioinhibition values at prolonged extraction
445 times (Supplementary Fig. 3b). Such dependence could be explained by transformations of more
446 toxic substances to less toxic products during the extractions with durations of 1 to 2 hours, followed
447 by migration of toxic compounds during prolonged extractions. Different temperature regimes do

448 not strongly affect the acute toxicity of solvent extracts, as the most toxic are extracts obtained after
1 449 hot solvent treatment (Supplementary Fig. 3c.). It should be mentioned that during microwave-
2 450 assisted extraction, the heat and mass gradients work in one and the same direction, contrary to the
3
4 451 other temperature regimes.
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6 452 The solvent-contact time interaction plot (Fig. 4.) shows the different behaviors of the
7 453 solvent extracts. The acute toxicity of the ethanol extracts increases with increasing extraction time,
8 454 since the toxicity of the aqueous extracts decreases. The acetic acid extracts follow the pattern
9 455 presented in the plot of the contact-time effect (Fig. 4a). The solvent-temperature regime interaction
10 456 plot (Fig. 4b) confirms the small effect of temperature regimes on the acute toxicity of extracts, as
11 457 the only exception is the low bioinhibition value of ethanol extracts obtained after microwave
12 458 treatment.
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19 460 **Fig. 4.**
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21 461 22 23 462 3.2.2. XenoScreen YES/YAS

24 25 26 463 a) Cans

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28 464 The MANOVA implementation to determine the endocrine-disruption potential of the can-
29 465 lining extracts exhibits a clear difference between their estrogenic and androgenic disruption
30 466 potential. The extracts of all solvents possess higher androgenic disruption potential than estrogenic
31 467 disruption potential (Fig. 5.). Significant androgenic agonistic potential (ratio higher than 1 with
32 468 respect to control values) has acetic acid extracts followed by DMSO and ethanol extracts, since
33 469 water extracts possess significant androgenic antagonistic potential. The time-effect plot presented
34 470 in Fig. 6. confirms the difference between their estrogenic and androgenic disruption potential.
35 471 Similar to the acute-toxicity study of can-lining extracts, the time-effect plot notes the maximum
36 472 androgenic disruption potential values at 48 h.
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44 476 **Fig. 5.**
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48 478 **Fig. 6.**
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52 480 The temperature does not strongly affect the solvent extract endocrine-disruption potential
53 481 (Fig. 7.), as this effect is not significant for estrogenic and androgenic antagonistic potentials. It seems
54 482 that for both can studies related to acute toxicity and endocrine-disruption potential, the migration
55 483 of toxic compounds occurs predominantly at 25°C.
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Fig. 7.

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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.

521 Taking into account the MANOVA results, the influence of temperature on the endocrine-
1 522 disruption potentials of extracts could be discussed only for their androgenic disruption potential.
2 523 The impact of temperature on androgenic agonistic activity of extracts is not well outlined, but
3 524 extracts obtained at 65°C show a well-pronounced androgenic antagonistic maximum
4 525 (Supplementary Fig. 6.). It could be concluded that migration of compounds with high androgenic
5 526 antagonistic activity occur at 65°C and 336 h contact time.
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9 527 The solvent-time interaction plot (Supplementary Fig. 7.) confirms significant endocrine-
10 528 disruption potential of acetic acid (YAS-), DMSO (YES-) and saliva (YES-, YAS-) extracts. The highest
11 529 androgenic antagonistic activity of acetic acid extracts obtained after 336 h supports the previously
12 530 mentioned increase with time. The solvent-temperature interaction plot shows that the maximum
13 531 values of antagonistic disruption potential for the acetic acid, DMSO and saliva extracts are obtained
14 532 at 65°C (Supplementary Fig. 8.). It should be mentioned that both interaction plots show significant
15 533 estrogen and androgen antagonistic potential in the saliva extracts.
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21 535 3.3. Multivariate statistics

22 536 In the figures below (Supplementary Figs. 9-11), the graphical output of the cluster and
23 537 principal components analysis is shown. It must be kept in mind that the chemometric analysis aims
24 538 to assess the effect of the experimental conditions on the toxicity record of three different types of
25 539 packaging material. This assessment is based on the results of the polynomial model obtained by the
26 540 experimental design procedure. The important features are therefore the regression coefficient
27 541 showing the effects of various experimental conditions in different extraction media and for different
28 542 packaging materials.
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34 544 a) Cans

35 545 In Supplementary Fig. 9a-c, the results from the hierarchical clustering of the variables
36 546 (regression weights) and of the various toxicity tests applied to the packaging materials for cans in
37 547 different extraction media are shown. Additionally, the biplot of PCA for the relationship between
38 548 the factor loadings for two identified latent factors is presented. The same scheme is used for the
39 549 other two packaging materials – multilayered composite packaging and cups.
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42 550 Based on data presented in Supplementary Fig. 9a, it is evident that a close relationship
43 551 exists between the features assessing the “average” toxicity (a_0), the temperature effect (a_1) and the
44 552 concentration effect (a_3) for can packaging. The effect of the time of extraction (a_2) is not directly
45 553 related to the other impacts and influences of mixed effects with time involvement. It could be
46 554 concluded that the toxicity of the can extracts is subject to two major impacts—one related to
47 555 concentration and temperature, and the other, to the time of extraction. This pattern separation has
48 556 to be considered in relationship with the results shown in Supplementary Fig. 9b. All XenoScreen
49 557 toxicity tests show a high level of similarity and form a very homogeneous cluster. This might mean
50 558 that the effect of the extraction media is negligible. On the other hand, the Microtox test indicates
51 559 variability with respect to the extraction media: Saliva and ethanol are outliers showing some
52 560 resemblance to the XenoScreen tests, and water and DMSO are relatively similar to each other but
53 561 very different from the XenoScreen tests. In Supplementary Fig. 9c, the relationship previously
54 562 mentioned for the linkage between the regression coefficients as indicators for experimental factors
55 563 impact is proven: a_0 , a_1 and a_3 belong to one latent factor, and a_2 belongs to another.

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1 565 b) Multilayer composite packaging

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3 566 As seen in Supplementary Fig. 10a, the clustering of the regression coefficients for multilayer
4 567 composite packaging is slightly different from that of the cans. Here, the “average” toxicity weight a_0
5 568 is related to a_2 and a_3 (the time of extraction and concentration), and a_1 (temperature impact) differs
6 569 from the rest. This might mean that for multilayer composite packaging, the temperature impact is
7 570 more specific, as the time of extraction impact was for can packaging. This is entirely confirmed by
8 571 the biplot of factor loadings (Supplementary Fig. 10c) from PCA. The linkage between the different
9 572 objects of multilayer composite packaging tested for toxicity (Microtox and XenoScreen) in different
10 573 extraction media is the same as in the case of can packaging: non-specific and homogeneous (with
11 574 respect to extraction media) results with XenoScreen and more specific with Microtox. Again, testing
12 575 the toxicity using Microtox indicates significant differences for water, ethanol and saliva media and a
13 576 slight resemblance between acetic acid and DMSO media with XenoScreen testing.
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21 578 c) Cups

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23 579 The results for the cup packaging material are obtained from the results of fewer
24 580 experiments as already indicated. In Supplementary Fig. 11a, the clustering of a_0 and a_3 is obvious,
25 581 since a_1 and a_2 are joined together. This means that the concentration factor is significant for the
26 582 general toxicity, and the time and temperature factors form the other pattern of impact. The
27 583 principal component analysis (biplot diagram in Supplementary Fig. 11c) confirms this conclusion. In
28 584 Supplementary Fig. 11b, the non-specificity of the XenoScreen tests is indicated again. The Microtox
29 585 test is seriously affected by the ethanol media (an outlier), and the other two media tested (water
30 586 and acetic acid) are similar to each other but different from XenoScreen.
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36 588 3.4. Results of FTIR studies

37 589 The spectra are presented in the electronic supplementary materials to this paper
38 590 (Supplementary Figs. 16-18, respectively, for the spectra of can linings, multilayer composite
39 591 packaging and cups). Each spectrum of the lining after extraction with a given solvent (for given time
40 592 and temperature regime) was compared in reference to the spectrum of the “original” lining, i.e.,
41 593 before the extraction. The bands missing after the extraction are indicated with the blue arrows. The
42 594 new bands that emerged or were definitely enlarged (as a result of the extraction) are denoted with
43 595 the red arrows.
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47 596 FT-IR ATR spectra of the can lining confirm the chemical character of the polymer as an epoxy
48 597 resin mixture (Supplementary Fig. 12). They also exhibit a very high degree of resemblance to the FT-
49 598 IR spectra of the diglycidyl ether of the bisphenol A mixture (Supplementary Fig. 13). The correlation
50 599 coefficients between the measured and library spectra are 67.41% for the epoxy resin and 67.37% for
51 600 the spectrum of the diglycidyl ether of the bisphenol A mixture. The spectral similarity was also
601 inspected visually. The major differences, i.e., additional bands that are found in the 2700-2100 cm^{-1}
602 region, are most likely caused by the contamination of the resin during the manufacturing process.
603 This additional absorption may be produced by the remnants of a catalyst (most probably
604 thiocyanate) in the polymer. Instrumental studies aiming for its identification are being continued. As
605 observed in all extraction experiments, the impurities responsible for the appearance of the

606 additional bands were repeatedly eluted with every solution applied to the extraction
1 607 (Supplementary Figs. 16a-y). This means they are not incorporated into the polymer chain but are
2 608 only weakly interacting with it. Moreover, it is very likely that these impurities are water-soluble.
3
4 609 Such conclusions are confirmed by the fact that the correlation percentage between the spectrum of
5 610 the epoxy resin and the studied can lining increased to nearly 71% after the extractions
6
7 611 (Supplementary Fig. 16).

8 612 The multilayer composite packaging lining is a pure polyethylene (93.99% resemblance,
9 613 Supplementary Fig. 14), which is basically not affected by the treatment with various solutions up to
10 614 65°C. At higher temperatures, the polyethylene is probably oxidized and/or absorbs the organic
11 615 molecules of the solvent, which results in the appearance of additional bands that are ascribed to the
12 616 absorption by carbonyl groups (please refer to Supplementary Fig. 17 for details). Similar to
13 617 multilayer composite packaging the cup lining is polyethylene (93.91% match, Fig. 15). The
14 618 correlations between the spectra of samples of cups treated with the studied liquids and the
15 619 spectrum of polyethylene remain within a very narrow range of 93.64-94.18%. The results indicate
16 620 that the interior of the cup is practically unaffected by treatments with the test solutions (please
17 621 refer to Supplementary Fig. 18 for details).

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23 623 4. Conclusions

24 624 The massive production and extensive usage of various packaging materials such as cups,
25 625 cans, and composites (e.g., TetraPak) require a careful assessment of the impact of different storage
26 626 and packaging conditions (temperature, disinfection, preserving, extracting and washing solvents) on
27 627 the pollution and toxic effects of the packaging that becomes waste. Since the variety of
28 628 temperature, type of solvent and concentration influences is quite broad, it seems reasonable to
29 629 interpret the experimental results by chemometric means, including carefully designed experiments.
30 630 An additional problem is the involvement of different ecotoxicity tests to assess the possible
31 631 hazardous impact of the packaging wastes after usage or in the case of prolonged food-package
32 632 contact under unfavorable conditions. Finally, an objective comparison of the chemical composition
33 633 of the package lining before and after extraction procedures seems important in order to correctly
34 634 assess the toxicity hazards.

35 635 FT-IR ATR spectroscopy is a useful, routine technique that allows to monitor the chemical
36 636 changes of a polymer coating without any special pretreatment of the samples, provided they adhere
37 637 to the refracting crystal (Tiefenhaller et al., 2017, Nikafshar et al., 2017, Yao et al., 2017). As studied
38 638 by Tiefenhaller et al. (2017) the thermal stability of diglycidyl ether of the bisphenol A varies greatly
39 639 with thermal regime applied and FTIR studies are indispensable to study processes occurring there
40 640 and versatile transformation products are generated in the process and released to surrounding
41 641 environment. Manguia-Lopez and Soto-Valez (2001) studied degradation and migration processes on
42 642 tuna fish and pepper cans with chromatographic and FTIR methods. Enhanced migration of BPA and
43 643 BADGE derivatives was stated in case of thermal treatment of objects (impact of time was not so
44 644 significant). FCM and food itself processing certainly has also impact the package material
45 645 degradation but also on degradation of (on the other hand favorable and desired) antioxidants; such
46 646 non-intentional impact of treatment and processing was studied e.g. by Alin and Hakkarainen (2011)
47 647 with gas chromatography methods or Gulmine et al. (2003) with FTIR spectroscopy. The microwave
48 648 heating (in comparison to conventional heating) caused significantly higher specific migration of
49 649 antioxidants studied. In other works, Atek and Belhaneche-Bensemra (2005) studied (with FTIR

650 methods) impact of simulation media and time/temperature regime on stability of poly (vinyl
1 651 chloride) as well as specific migration; the applicability of spectroscopic methods was also confirmed
2 652 in field of mass transfer studies. For these, among other, reasons Nikafshar et al. (2017) started
3 653 searching for renewable bio-based epoxy resin that could possibly be used as can lining material
4 654 however with reduced toxicity. The vanillin-based product was confirmed to possess similar
5 655 mechanical behavior like currently used analogs of diglycidyl ether of the bisphenol A.

6 656 Utilizing biological methods in FCM degradation and SM studies is less common than
7 657 application of instrumental techniques known since mid XIX century. Most of these biological studies
8 658 involved acute toxicity, endocrine potential, genotoxicity, cytotoxicity and mutagenicity
9 659 determination. In the work of Mertl et al. (2014) migration of xenobiotics from carton and
10 660 polypropylene packaging into food simulants was studied with CALUX assay; antagonistic effects
11 661 were noted for foil and carton for milk product extract while no androgenic actions could be noted.
12 662 Endocrine potential of recycled and virgin paper kitchen rolls was also studied by Vinggaard et al.,
13 663 (2000). In this case estrogenic response was observed mainly for ethanol extracts of recycled paper.
14 664 Test with HELN, HG5LN cell lines was used by Chevolleau et al. (2016) to assess toxicity/endocrine
15 665 potential of water stored in PET and glass bottles under different treatment processes, although the
16 666 approach suggested did not allow to obtain any distinctive measures of endocrine potential. Such
17 667 potential was found by Wagner and Oehlmann (2011) that performed similar studies however
18 668 utilizing E-SCREEN assay; significant estrogenic effects in a human cancer cell line from 19.8 to 50.2%
19 669 compared to 17-estradiol was confirmed. Galotto and Ulloa (2010) studied estrogenic activity of
20 670 simulated liquids extracts of plastic packaging. Fatty simulation products appeared to have the
21 671 highest estrogen activity for paper/aluminum/PE composites. Acute toxicity with bioluminescent
22 672 bacteria of extracts of metal cans with polymeric linings was studied by Szczepańska et al. (2017) and
23 673 acetic acid extracts appeared to be the most toxic to bioindicating organisms.

24 674 Certainly more studies are being performed in this area however their description falls out of
25 675 scope of this paper. Still, it should be stressed that none of approaches utilized all three
26 676 (instrumental, biological and chemometric) methods to study degradation phenomena of FCM. In the
27 677 present studies, all of these aspects mentioned above were taken into account by the application of
28 678 a full factorial experimental design followed by i) regression modeling of the experimental effects on
29 679 the ecotoxicity readings, ii) ANOVA and MANOVA estimation of the experimental conditions as
30 680 significant factors affecting the toxicity results and iii) IRFT analysis of the packaging. It is shown that
31 681 the effects of temperature and extraction solvents could be quantitatively assessed with good
32 682 correlation between different statistical estimates. Comparison of the results of instrumental and
33 683 biological studies confirm the assumptions that bioassays can serve as a valuable and validated tool
34 684 in assessing the stability and impact of packages on goods stored both for short and long periods of
35 685 time. In some cases, the bioassays were proven to be more sensitive and reliable than IR studies,
36 686 especially when studies deal with assessing the impact of acidic or alcohol-containing stimulants. In
37 687 this study, it was confirmed that instrumental screening studies are not precise enough to assess the
38 688 change in composition of FCM after some basic treatment processes, reflecting those processes that
39 689 naturally occur during packaging and storage. On the other hand, bioassays may deliver suitable
40 690 information when the results of extract screening of materials are referred to validated results for
41 691 their respective lining material and chemometric tools are utilized to assess these relationships.
42 692 Certainly, the method described, although proven to be reliable tool in FCM studies, has its
43 693 limitations; just to mention: i) necessity to use non-toxic simulation agents not always reflecting

694 already standardized instrumental methodologies, ii) need of performing studies with certifies
1 695 organisms from different trophic levels, iii) lack of sufficient number of correlation studies to explain
2 696 observed toxicological responses under different extraction conditions, iv) involving time-tested and
3 697 sometimes highly qualified personnel to perform instrumental, biological and chemometric studies.
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6 698 In any case, the performance of future work is indispensable to i) identify the chemicals
7 699 responsible for observable toxicological levels (currently being performed in many scientific centers
8 700 and laboratories including those of the authors), ii) assess the applicability of other bioassays as
9 701 presented in the methodology of this research, iii) construct a battery of bioassays enabling exposure
10 702 and degradation assessment for different types of packages and FCM, being at the same time
11 703 complementary and universal for various treatment regimes, iv) propose international standards on
12 704 the application of certified bioassays as recognized tools in the assessment of exposure to pollutants
13 705 migrating from and created within FCMs, and v) comprehensively estimate (with classical and
14 706 biological tools) the impact of packaging material wastes reaching different environmental niches
15 707 after being used by households and industry, as they pose a serious source of small molecules and
16 708 microplastics.
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832 **Figures captions:**

1
2 833 Fig. 1. Schematic presentation of research performed in the study

3
4 834 Fig. 2. Solvent interaction effect plots for acute toxicity determination of can lining extracts with: (a)
5 835 contact time [h] and (b) temperature [°C]

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7 836 Fig. 3. Solvent interaction effect plots for acute toxicity determination of multilayer composite
8 837 packaging lining extracts with: (a) contact time [h] and (b) temperature [°C]

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11 838 Fig. 4. Solvent interaction effect plots for acute toxicity determination of cup internal surface extracts
12 839 with: (a) contact time [h] and (b) temperature regime

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15 840 Fig. 5. Solvent effect plot for endocrine disruption potential determination of can lining extracts

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17 841 Fig. 6. Time effect plot for endocrine disruption potential determination of can lining extracts

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19 842 Fig. 7. Temperature effect plot for endocrine disruption potential determination of can lining extracts

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21 843 Fig. 8. Solvent-time interaction plot for endocrine disruption potential determination of can lining
22 844 extracts

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24 845 Fig. 9. Solvent-temperature interaction plot for endocrine disruption potential determination of can
25 846 lining extracts



Figure 1

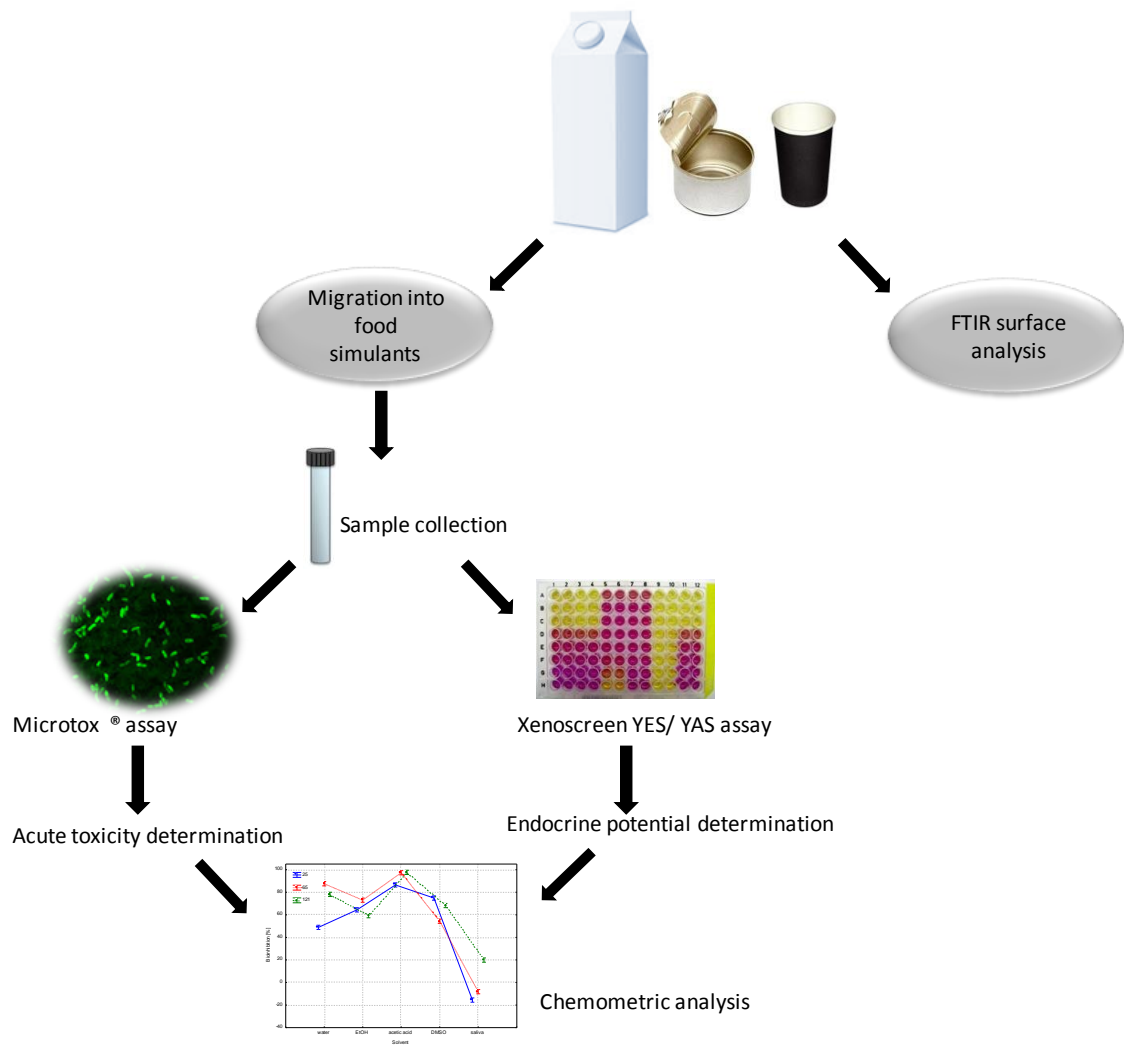


Fig. 1. Schematic presentation of research performed in the study

Figure 2

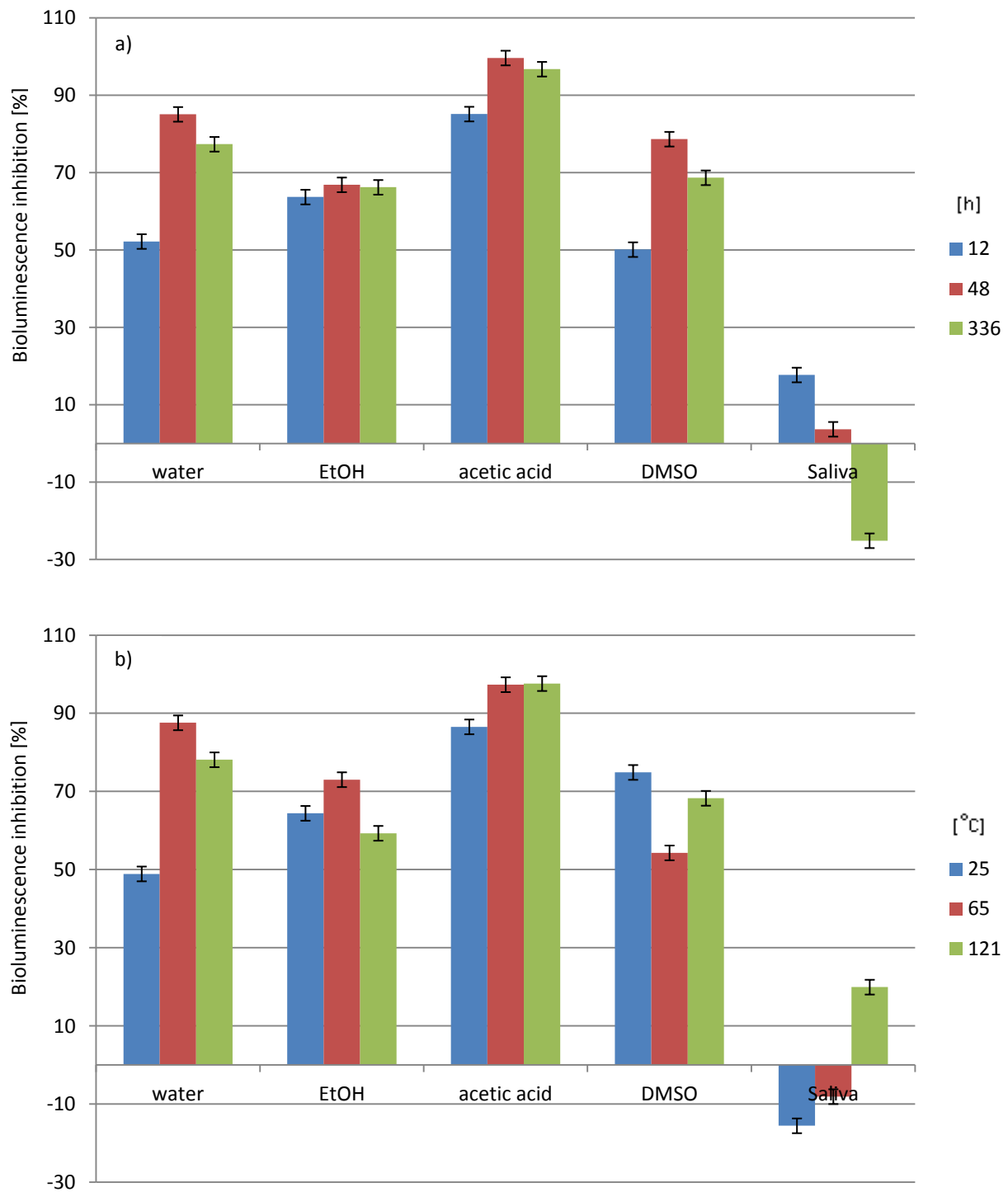


Fig. 2. Solvent interaction effect plots for acute toxicity determination of can lining extracts with: (a) contact time [h] and (b) temperature

Figure 3

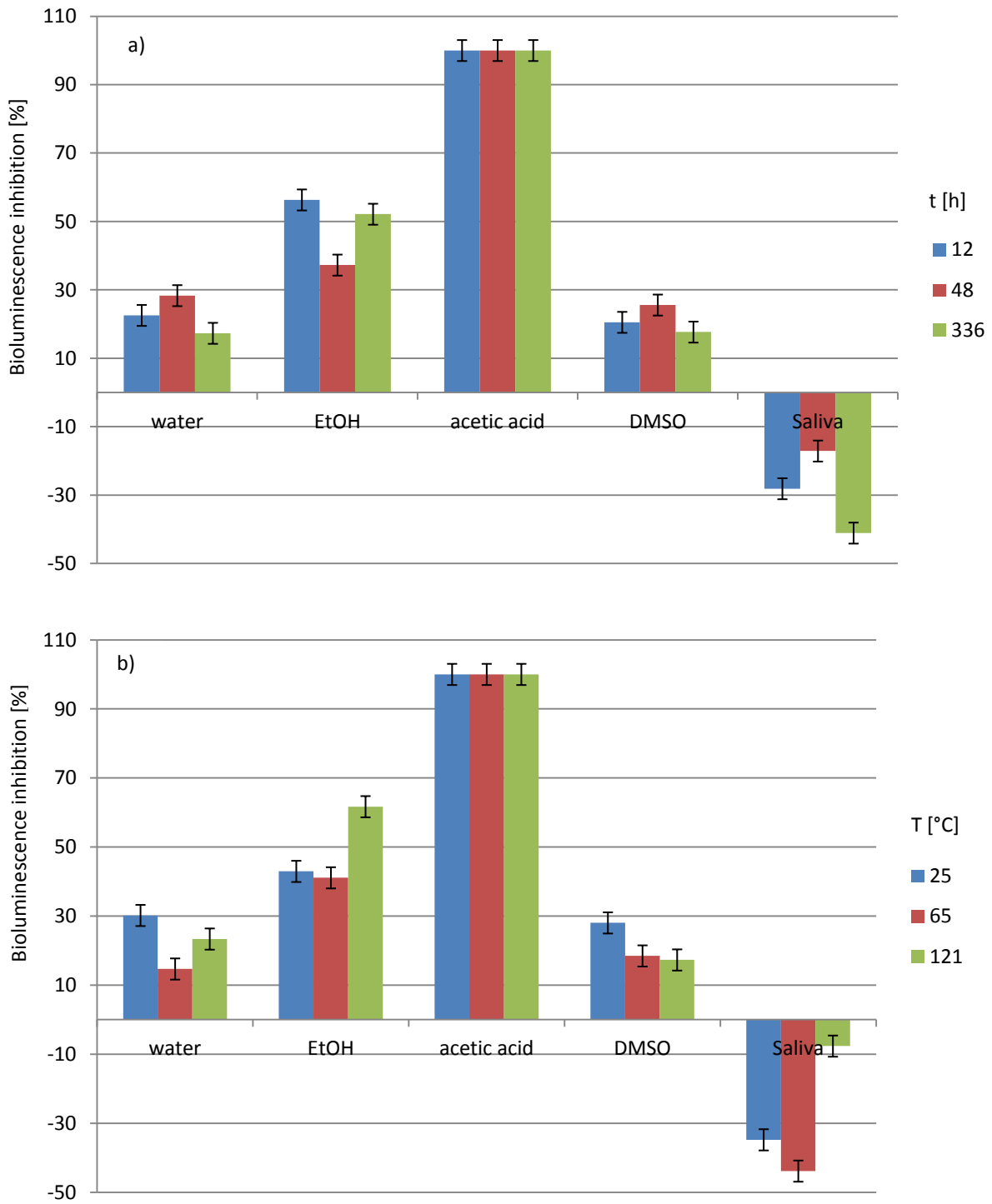


Fig. 3. Solvent interaction effect plots for acute toxicity determination of multilayer composite packaging lining extracts with: (a) contact time [h] and (b) temperature [°C]

Figure 4

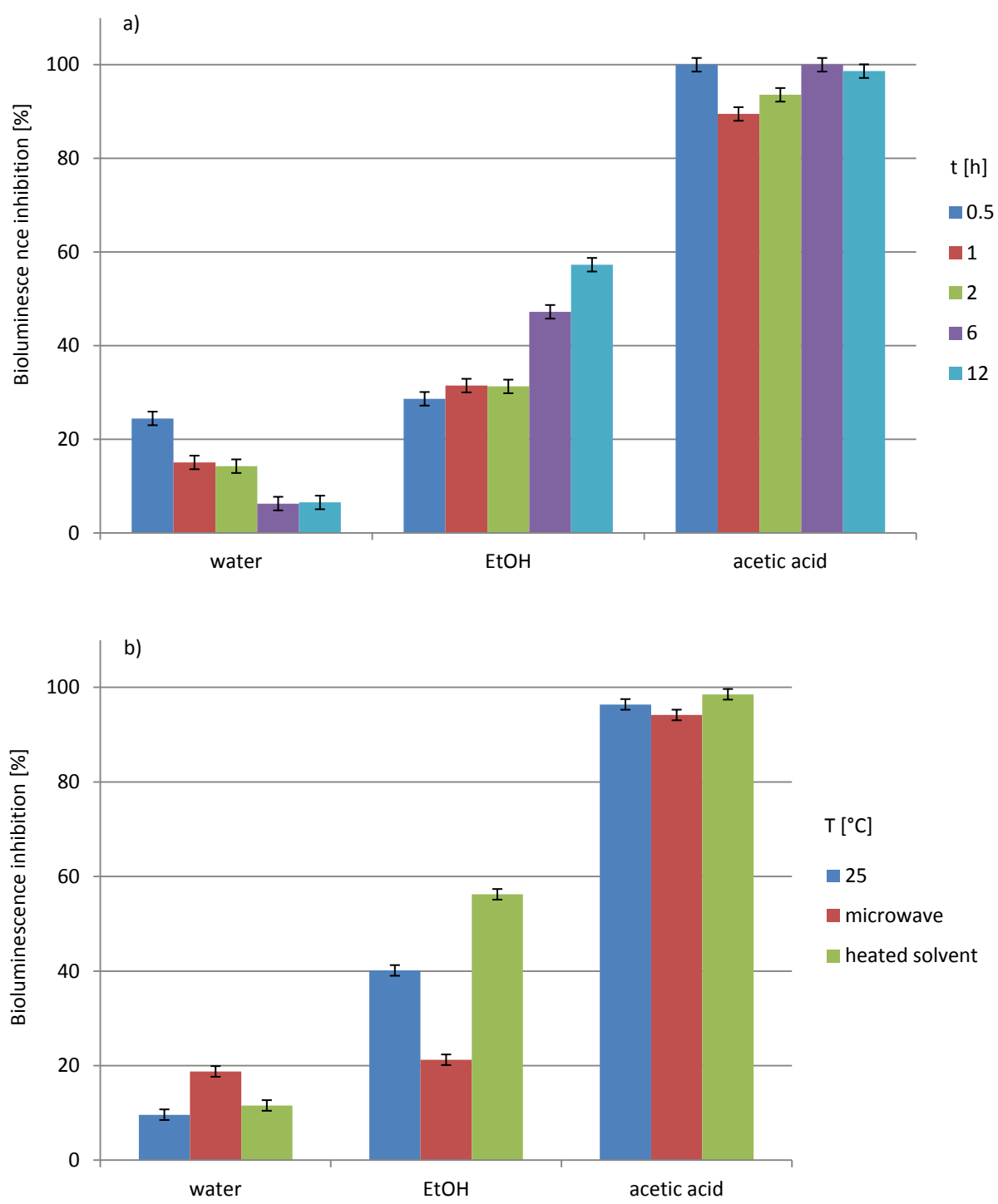


Fig. 4. Solvent interaction effect plots for acute toxicity determination of cup internal surface extracts with: (a) contact time [h] and (b) temperature regime

Figure 5

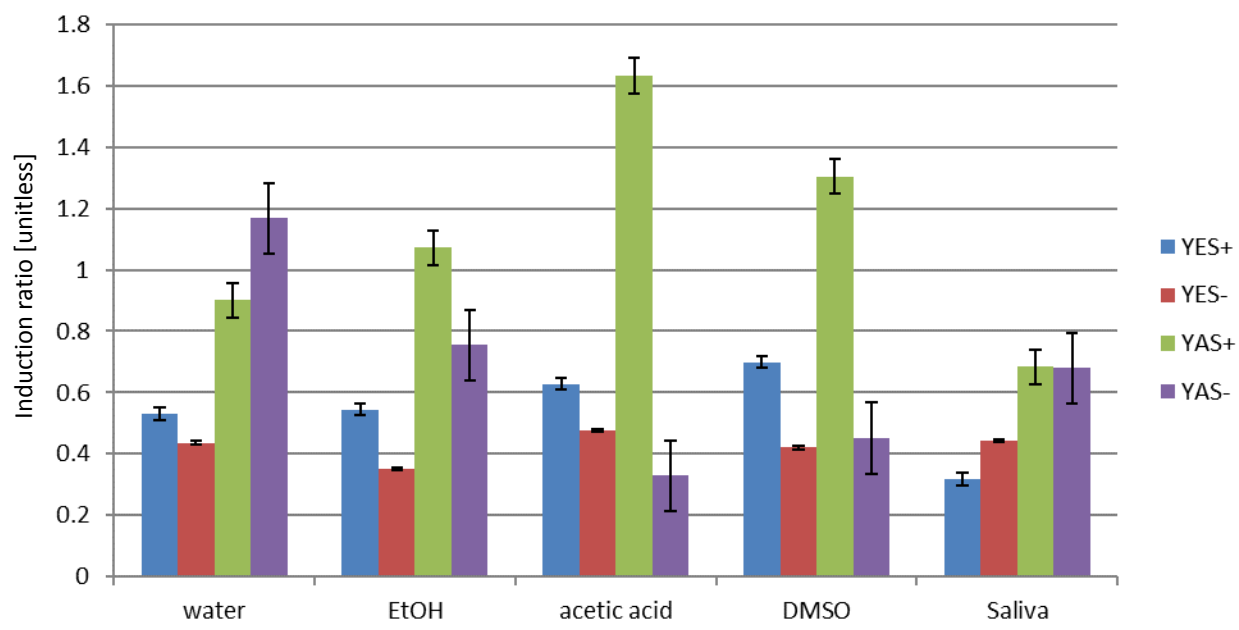


Fig. 5. Solvent effect plot for endocrine disruption potential determination of can lining extracts

Figure 6

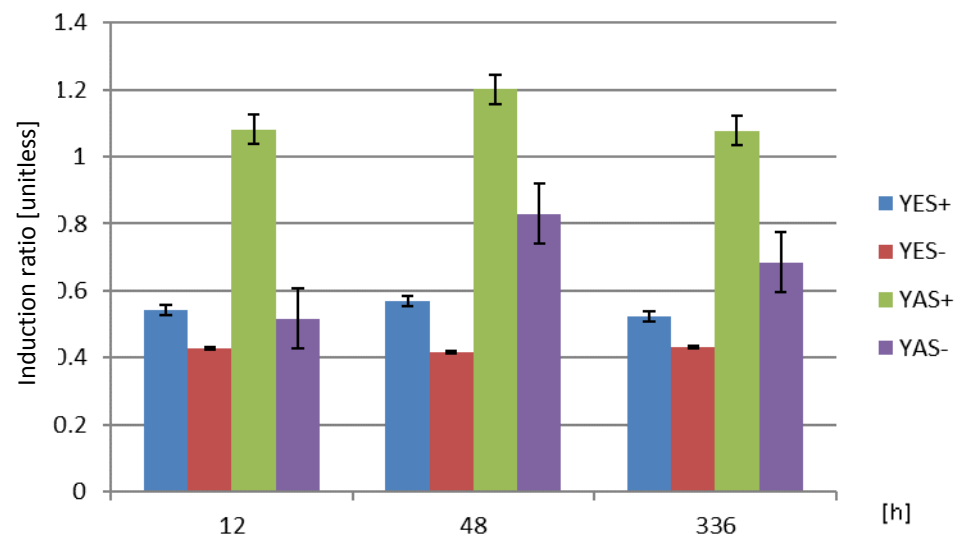


Fig. 6. Time effect plot for endocrine disruption potential determination of can lining extracts

Figure 7

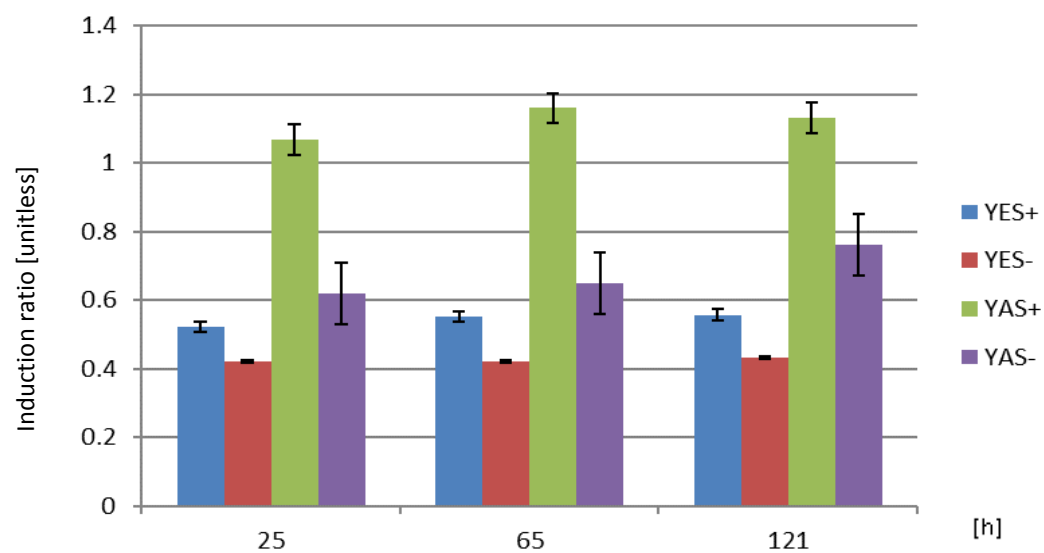


Fig. 7. Temperature effect plot for endocrine disruption potential determination of can lining extracts

Figure 8

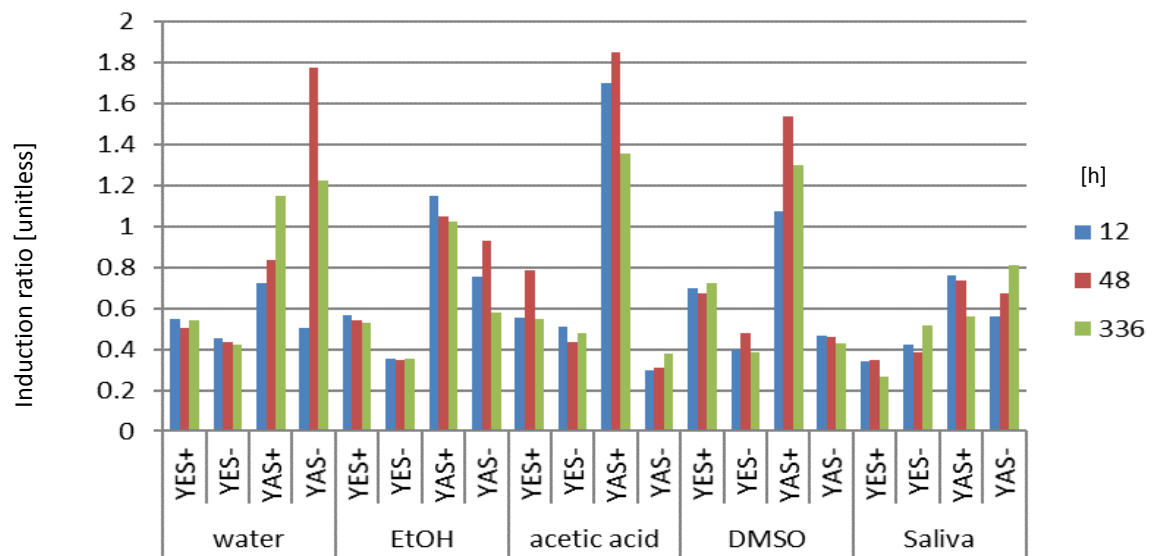


Fig. 8. Solvent-time interaction plot for endocrine disruption potential determination of can lining extracts

Figure 9

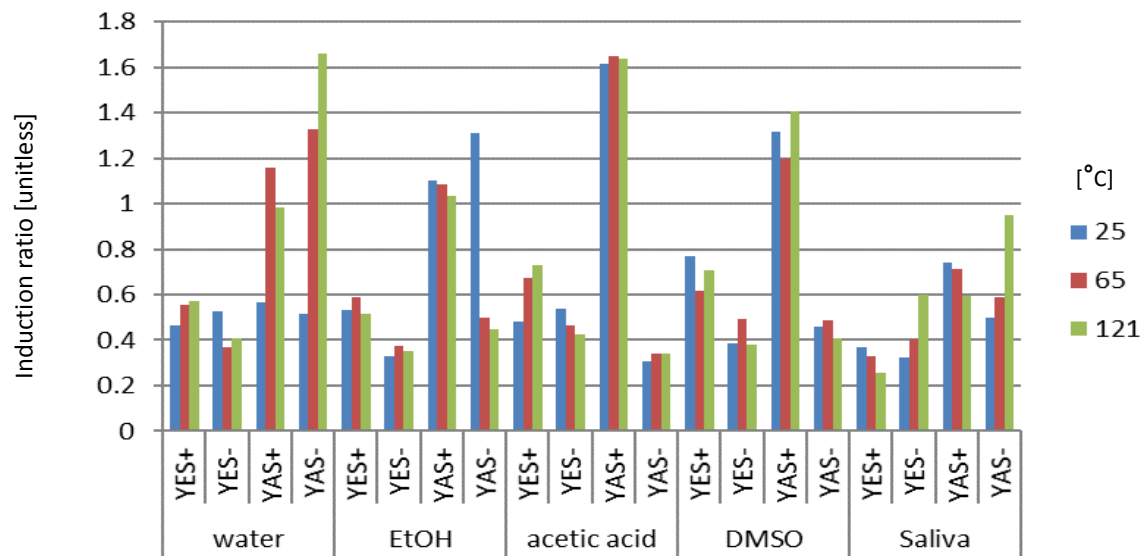


Fig. 9. Solvent-temperature interaction plot for endocrine disruption potential determination of can lining extracts