Assessing Acute Toxicity of Selected Packages Internal Layers Extracts using Microtox®

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In the scientific literature related to the widely understood issue of packaging materials designed to have contact with food (food contact materials), there is much information on raw materials used for their production, as well as their physiochemical properties, types and parameters. Unfortunately, not much attention is given to the issues concerning migration of toxic substances from packaging and its actual influence on the health of the final consumer, even though health protection and food safety are the priority tasks. The goal of this study was to estimate the impact of particular foodstuff packaging type, food production and storage conditions on the degree of leaching of potentially toxic compounds to foodstuffs with the use of the acute toxicity test Microtox®. From all simulants studied, the 3% acetic acid in water proved to cause significant migration of toxic compounds with increase of time and temperature of extraction and justified the hypothesis that food products with low pH values (stored in cans) cause significant damage to cans internal resin filing and is a reason of increased migration rate of package material to foodstuff.

KEY WORDS: food packaging; extraction; migration; toxicity; biotest; Microtox®

INTRODUCTION

Scientific literature, describing the latest research findings related to the monitoring of xenobiotics and addressing different toxicological aspects, presents extensive information on the sources and volume of emissions released by everyday objects. The migration of low molecular mass compounds from food packaging to the food stored inside is a priority in this area of research. The multiannual research performed in numerous research centres enables the identification of compounds leaching from the inner layer of packaging as well as conditions that intensify this process.[1] Among the most commonly determined analytes in simulants, as well as in food samples stored in metal cans or in polymeric packaging materials, the following predominant can be distinguished: bisphenol A, bisphenol A diglycidyl ether (BADGE), their derivatives and phthalates.[2] The main sources of contamination are the special coatings used to protect the inner layer of the packaging from corrosion and to protect food from direct contact with the material used in producing the container. It is known that because of technological processing and interaction with food ingredients, migrating compounds may transform into various types of derivatives with physicochemical and toxicological properties different from the parent compound.[3]

The main criteria for approving packaging materials for coming into contact with food deal with the toxic properties of substances used to produce a given material and the degree of migration and specific migration determined based on normative values of tolerable daily intake. However, the standard guidelines for assessing the impact of packaging on the quality of food consider only the numerical values of 'overall migration' and 'specific migration' limits, which are parameters fixed for a small group of compounds.[4] With respect to metal packaging, xenobiotics originate mainly from the types of special coating used to protect the internal layer of the package against corrosion and to prevent direct contact between the food and the material from which the package was made.[3,5] The most common coatings are epoxy resins and vinylic organosols (also called polyvinyl chlorides). According to the Commission Regulation on the restriction of use of certain epoxy derivatives in materials (EC) No 1895/2005, the sum of BADGE·HCl, BADGE·2HCl and BADGE·HCl·H_2O must not exceed 1 mg/kg in food or food simulant, and the sum of BADGE and its hydrolysed derivatives must not exceed 9 mg/kg.[6]

This approach raises many objections. Results from a recent study show that apart from the monitored compounds leaching from the surface of the packaging, other contaminants and their derivatives are formed by interaction with food components and because of the technological procedures applied.[7,8] All unidentified contamination is described in the literature as *non-intentionally added substances*.[2] The inability to accurately identify and quantify all the substances released into food and the lack of adequate toxicological knowledge, make it impossible to assess the real danger to consumers and to predict the possible consequences associated with long-term exposure. Importantly, the vast majority of synthetic compounds used for the production of protective layers exhibits properties similar to endocrine disrupting compounds.[9] In the environment, the presence of compounds affecting proper functioning of the endocrine system poses many problems for researchers. For many years, numerous research centres have been conducting studies to explain the processes and mechanisms by which these compounds modify the functioning of the living organism. Unfortunately, knowledge about the complex mechanisms of toxicity of these compounds is very limited.[10] Furthermore, according to extensive research performed *in vitro* and *in vivo*, the dose–response for these compounds is not monotonic; hence, the data obtained by exposing animals to high doses of xenoestrogens to assess the risk of toxic effects in humans exposed to low doses over a longer period of time can be seriously flawed. Therefore, applying traditional principles and assumptions used to assess health risks for other contaminants cannot be used to evaluate hormonally active compounds.[11] Moreover, toxicological assessment of hormone derivatives should consider the interactions between these contaminants. The presence of several xenoestrogens at low or even minimal concentrations may result in a summarized toxic effect. The combined action of biologically active compounds may result in synergy, which reinforces the effects of toxicity or antagonism, which may reduce the toxic effects.[12] Clearly, it is not possible to estimate the risk posed by several xenobiotics occurring at different levels of content based on the composition of the sample alone. Determining the toxicity of the sample is only possible by applying methods that utilize living organisms as active elements. One of bioassays commonly used, standardized and well-known is Microtox® (using bacteria as an active element). Because of high sensitivity of bioluminescent organisms to toxic substances, the biotest is widely used in environmental research. Currently, it is used as a tool for the assessment of toxicity of water bodies, bottom sediments and soils' condition, to assess the efficiency of sewage treatment by wastewater treatment plants. A relatively low cost of analysis and fast results were the reasons for using this test not only in standard environmental research but also more and more frequently to assess the toxicity of medicines,[13] new materials such as ionic liquids[14] or nanoparticles.[15] The test is also applied to assess the toxicity of body fluids, which may contribute to the development of methodologies enabling fast detection of many diseases.[16]

Currently, there is little information on the cumulative negative effects of small molecule components of food packaging. Microtox® has been used to assess toxicity of extracts because microorganisms represent the primary focus in the food chain; therefore, any adverse changes occurring in them, directly or indirectly, can have impact on organisms at higher trophic levels. The results reviewed in this study will stimulate the development of new approaches for production of safer packaging materials. The objective of this study was to estimate the impact of different conditions of storage on the degree of leaching of potentially toxic compounds from internal layers of metal cans.

MATERIALS AND METHODS

Samples collection

A review of the literature data indicates that factors that have the biggest influence on the intensity of the process of migration of contamination from the packaging material are the temperature, contact time and physiochemical properties of the medium simulating product stored.[3] Twenty four metal cans devoted for fish storage were obtained from local producer in Ustka (Poland). To our best knowledge, the interior layer of packaging was covered with epoxy resin. Because one of the aims of the study was to assess impact of different simulation media on the degradation rate of package internal layer, it was decided to use more simulation media then suggested by the purpose of package. Three simulants respectful to specific kinds of food have been chosen in relation to the methodologies used in order to assess global migration, as described in Commission Regulation (EU) No 10/2011: distilled water for aqueous foods with a pH above 4.5; acetic acid at 3% in distilled water for acidic aqueous food with pH below 4.5; ethanol at 5% for any food that may contain alcohol (standard procedures e.g. in the EU legislation recommend using 10% EtOH however because of high alcohol content, there was observed unintended toxicity increase and lower ethanol content had to be utilized). Additional medium – dimethyl sulfoxide (DMSO) – was used in regard to the possibility of using it as an simulation medium. The simulation media were selected to be benign to bacteria and in reference to aforementioned standards. Xenobiotics migration from epoxy resins was studied at three different temperatures (room temperature = 25°C, 65°C and 121°C); selected food cans were doubly heated to 65°C and 121°C after 5 and 10 days prior to start of the migration process (to study impact of such process on degradation of cans' internal resin layer and release of contaminants to different simulation media). Selection of proper temperature regime was carried out in accordance with EU Regulation because canned food is sterilized in 121°C for 30 min. Heating to 65°C aimed at checking whether or not such conditions of incubation affects release of xenobiotics in any way. The cans were filled with 130 ml (5 mm below the edge) of simulant and the heating process (t = 30 min) was applied. Thirty millilitre sample aliquots were taken after 12 and 48 h and 2 weeks, in order to assess the impact of time of contact between the simulation medium and the polymer layer on the degree of leaching the toxic compounds. pH was set to fall within the 6.5–7.5 range with concentrated HCl/NaOH solutions (to assure optimal bacterial growth conditions).

Chemicals and reagents

Chemicals that were used for preparing simulation media were obtained as follows: ethyl alcohol, hydrochloric acid (HCl), sodium hydroxide (NaOH) (POCh S.A., Poland), acetic acid (35–38% w/w) (Chempur, Poland), DMSO (Sigma Aldrich, Germany) and distilled water. Chemicals used for Microtox® were purchased from Modern Water Ltd. (Modern Water Ltd., USA). These were 2% NaCl solution, lyophilized Vibrio fischeri, Microtox Diluent, Microtox Acute Reagent, Osmotic Adjusting Solution and Reconstitution Solution. All reagents were of analytical grade purity or better in case of reagents for microbiological purposes. The instruments and equipment used during the studies were Microtox® 500 analyser of Modern Water Ltd. (Modern Water Ltd., USA), electronic pipettes (Mettler Tolero, USA, Eppendorf, Germany), heater of Thermicon P® type K1253S (Heraeus Instruments, Germany), water bath shaker type 357 (Elpan Laboratory Instruments (Poland) and CP411 Metron pH-meter (Metron, Poland).

Microtox® assay

Acute toxicity was assessed by determining the luminescence inhibition of the marine Gram(−) bacteria *V. fischeri*, after a 30 min exposure to different 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. In Figure 1a, diagram of the Microtox® test procedure used during the research is presented. The data were processed using the MICROTOX OMNI SOFTWARE, according to the Basic Test Protocol (81.9%).

For each food simulant two independent variables, factorial design was performed (refer to Table 1.).

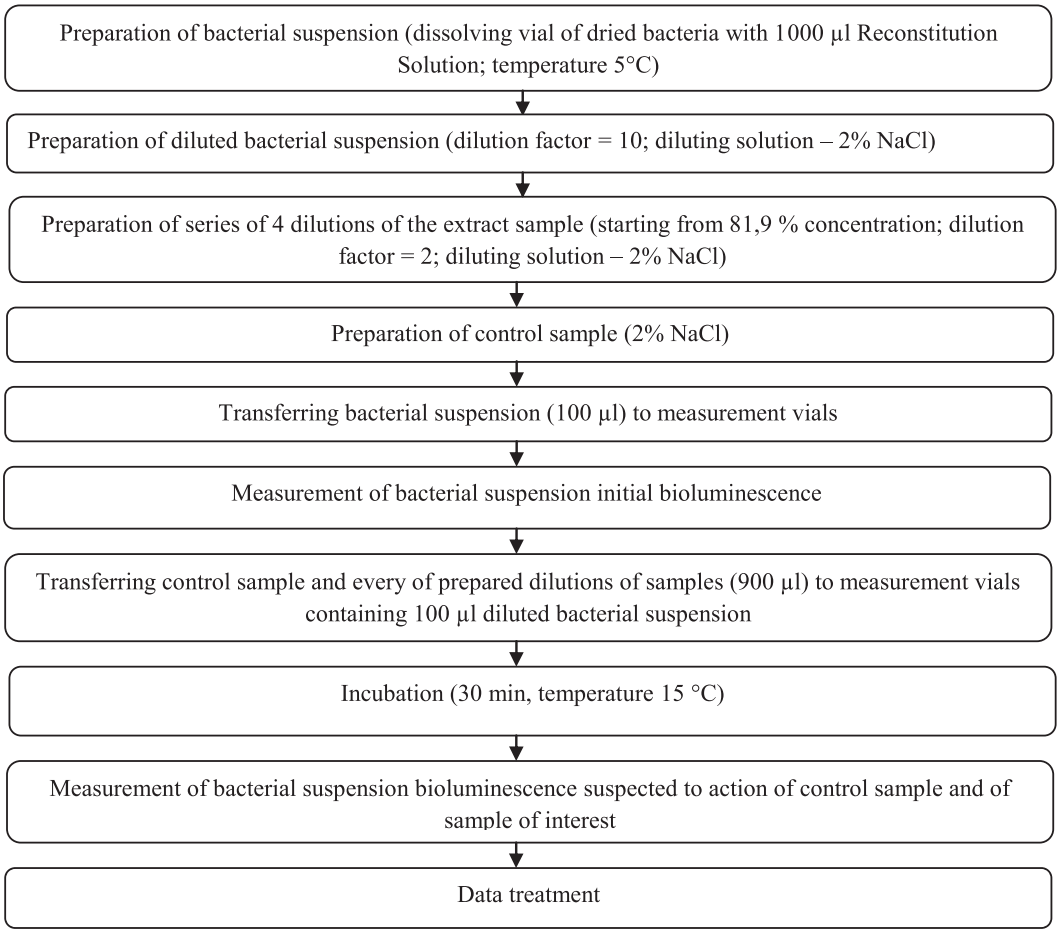

Figure 1. Analytical methodology of acute toxicity determination in the studies presented.

Table 1. Factorial experimental design.

	Factor 1 (temperature)		
Factor 2 (contact time)	25°C	65°C	121°C
12 h	25°C and 12 h	65°C and 12 h	121°C and 12 h
48 h	25°C and 48 h	65°C and 48 h	121°C and 48 h
336 h	25°C and 336 h	65°C and 336 h	121°C and 336 h

Each of both variables (factors) – temperature and contact time – took three levels. Each combination of levels for the independent variables creates a certain experimental conditions. 3 × 3 factorial design creates nine treatment conditions as each experiment was run in triplicate.

Statistical analysis

Multifactor analysis of variation (MANOVA) was used to evaluate the effects of the two factors (temperature and contact time) and their interaction on the dependent variable (acute toxicity) for four different simulation solvents (distilled water, ethanol, DMSO and acetic acid). The two way MANOVA procedure compares the acute toxicity results obtained at different experimental conditions for each food simulant.

RESULTS AND DISCUSSION

Mean bioluminescence inhibition values for all experimental conditions (and in relation to toxicity values of pure simulants) are presented in Table 2 [the results of toxicity determination with 81.9% Basic Protocol for pure simulation media were $EC_{50,\ acetic\ acid}$ = 27.56%, 164 $EC_{50,\ ethanol}$ = 76.40% and $EC_{50,\ DMSO}$ 6.65% (highest observable toxicity) for acetic acid, ethanol and DMSO, respectively]. Because in case of experiments with acetic acid calculation of the EC_{50} values was almost impossible (because of high toxicity of simulation media), it was decided that bioluminescence inhibition value at 89.10% concentration level should be taken into consideration for MANOVA (to indicate parameters resulting in high toxicity values being observed). Furthermore, the bioluminescence inhibition values predicted by MANOVA model will be presented and discussed.

The MANOVA model for evaluation of experimental data for distilled water as a food simulant exhibits significant influence of temperature, contact time and their interaction on acute toxicity. For both independent variables (temperature and contact time), acute toxicity increases (increase of bioluminescence inhibition) with increase of independent variables (Figure 2a and b). The negative value (increase of bioluminescence) at the lowest temperature (25°C) and contact time (12 h) may suggest that hormesis occurs at corresponding experimental conditions. The difference between acute toxicities at higher independent variable levels is not significant, which means that significant leaching of chemicals after 65°C and 48 h does not occur. The last conclusion (easily seen on interaction effect plot, Figure 2c) proves that the significance of interaction effect is caused by favourable conditions for bacteria at 25°C and 12 h duration of extraction procedure.

The MANOVA model (for ethanol used as simulant) shows significance of all three effects. For temperature and contact time, the maximum mean values are at their medium levels (65°C and 48 h) with maxima more pronounced at temperature plot (Figure 3a and b). It seems that at these levels, migration of toxic compounds occurs, which lead to adverse conditions for bacteria and increase of inhibition of bioluminescence. The curves for 12 and 48 h in interaction plot (Figure 3c) have the similar shape to temperature 1 because at 2 weeks experiments there is sharp decrease of inhibition of bioluminescence values with increase of temperature. The possible reason for this decrease of acute toxicity is chemical transformation of extracts' ingredients that lead to less toxic chemical species. Available data indicate that BADGE may be easily hydrolysed when in contact with aqueous or acidic media with formation of the secondary compounds such as BADGE·H_2O, BADGE·$2H_2O$ and BADGE·2HCl.[17] Results of studies of Sueiro *et al.* (2006) justify the statement that BADGE shows much higher mutagenic effect to bacteria than BADGE·H_2O. No mutagenic activity was found for BADGE·$2H_2O$ and BADGE·2HCl.[18] It can be concluded that observed reduction of toxicity is due to transformation of primary compounds to less toxic secondary chemical (which requires further instrumental studies). Still it seems required to conduct next model studies to assess summarized toxic effect of all ingredients of extracts obtained.

Table 2. Results on toxicity determination of food packages extracts (medium bioluminescence inhibition values in [%] of n = 3 three experiments with respective standard deviation in brackets).

	Contact time [h]	Temperature [°C]		
		25	65	121
Water	12	−13.4 (0.02)	90.7(0.02)	79.3 (0.04)
	48	98.3 (0.02)	82.8(0.03)	74.1(0.03)
	336	61.8 (0.01)	89.2 (0.01)	81.0 (0.01)
Ethanol	12	54.9 (0.02)	77.6 (0.03)	58.6 (0.01)
	48	54.0 (0.01)	75.0 (0.03)	71.5(0.02)
	336	84.4 (0.02)	66.5 (0.02)	47.8 (0.02)
Dimethyl sulfoxide	12	66.3 (0.03)	29.0 (0.04)	55.1(0.02)
	48	85.2 (0.06)	68.7 (0.04)	82.1 (0.11)
	336	73.2 (0.01)	65.2 (0.04)	67.6 (0.02)
Acetic acid	12	59.6 (0.01)	100 (0.00)	95.8 (0.02)
	48	100 (0.00)	100 (0.00)	98.8 (0.01)
	336	100 (0.00)	92.0 (0.01)	98.2 (0.01)

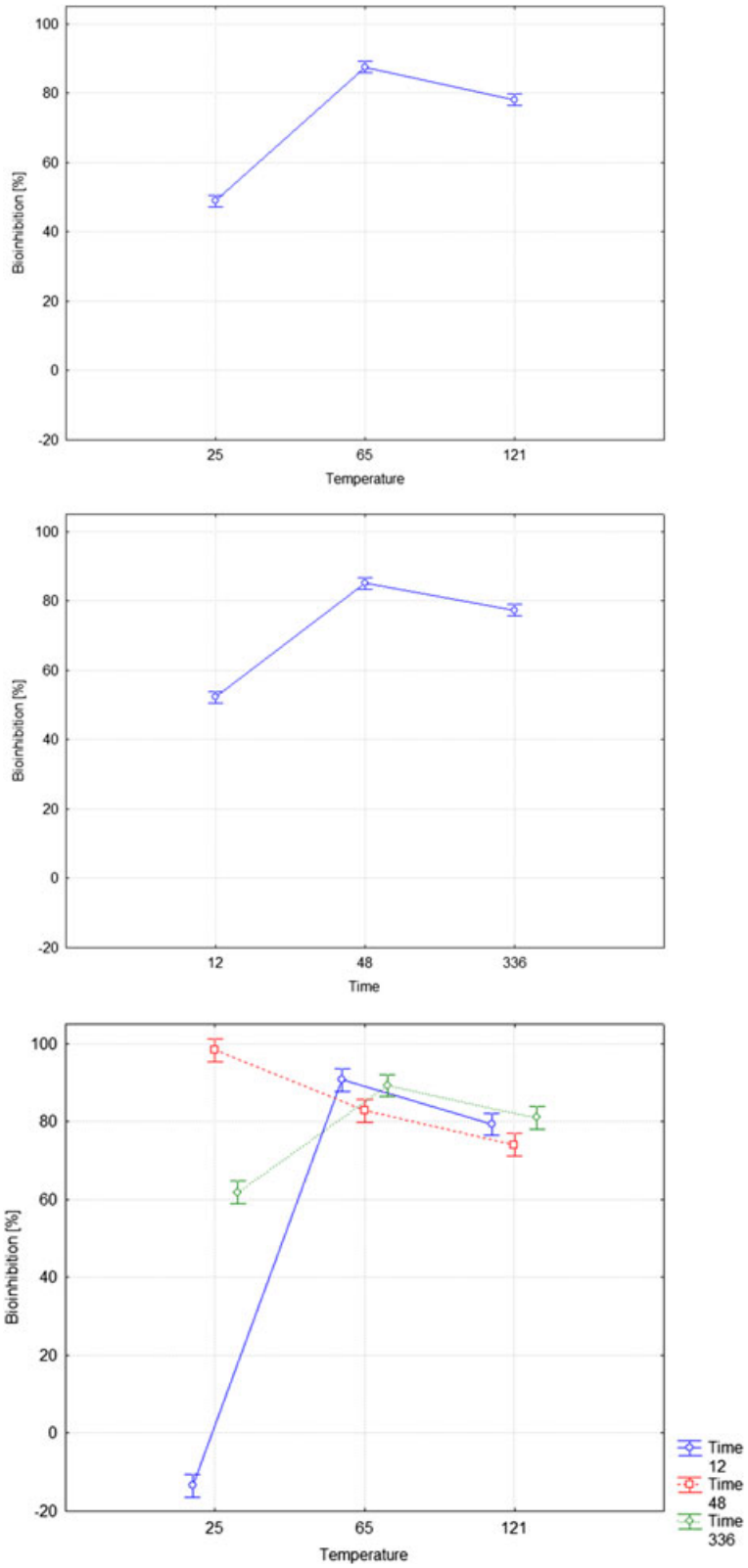

Figure 2. Effect plots for water extraction experiments: (a) temperature [°C], (b) contact time [h] and (c) interaction temperature–contact time (points are shifted aside for better perspicuity).

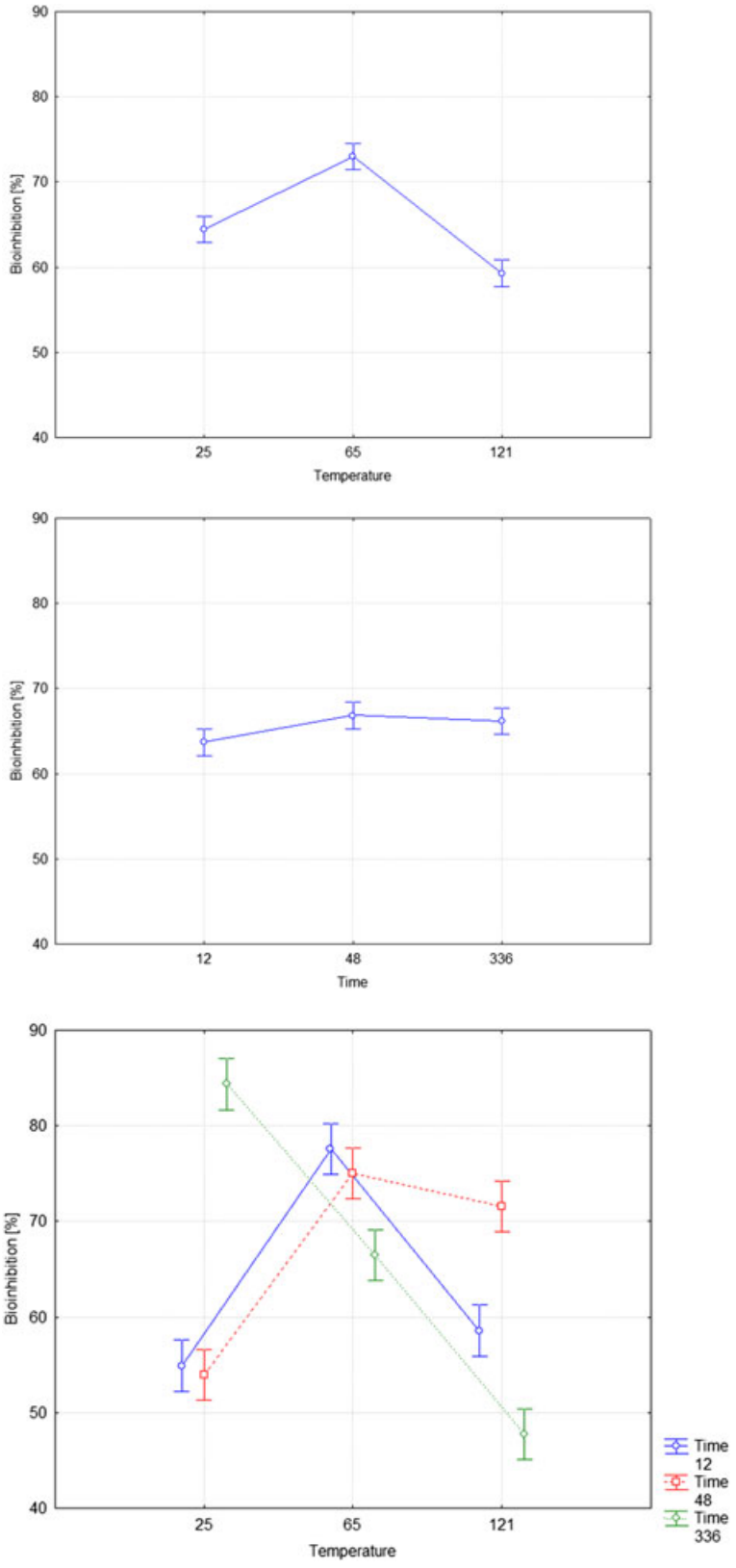

Figure 3. Effect plots for ethanol extraction experiments: (a) temperature [°C], (b) contact time [h] and (c) interaction temperature–contact time (points are shifted aside for better perspicuity).

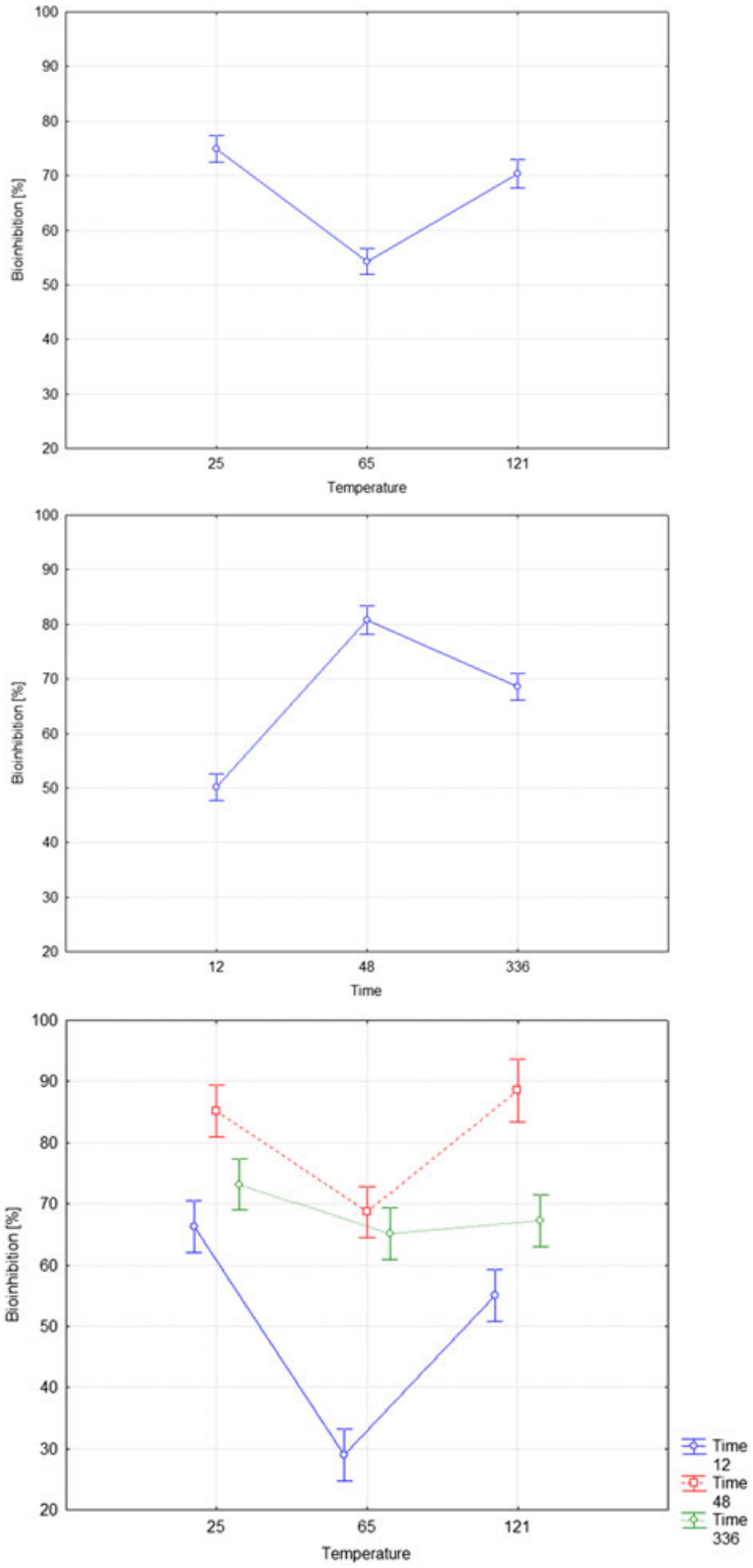

Figure 4. Effect plots for DMSO extraction experiments: (a) temperature [°C], (b) contact time [h] and (c) interaction temperature–contact time (points are shifted aside for better perspicuity).

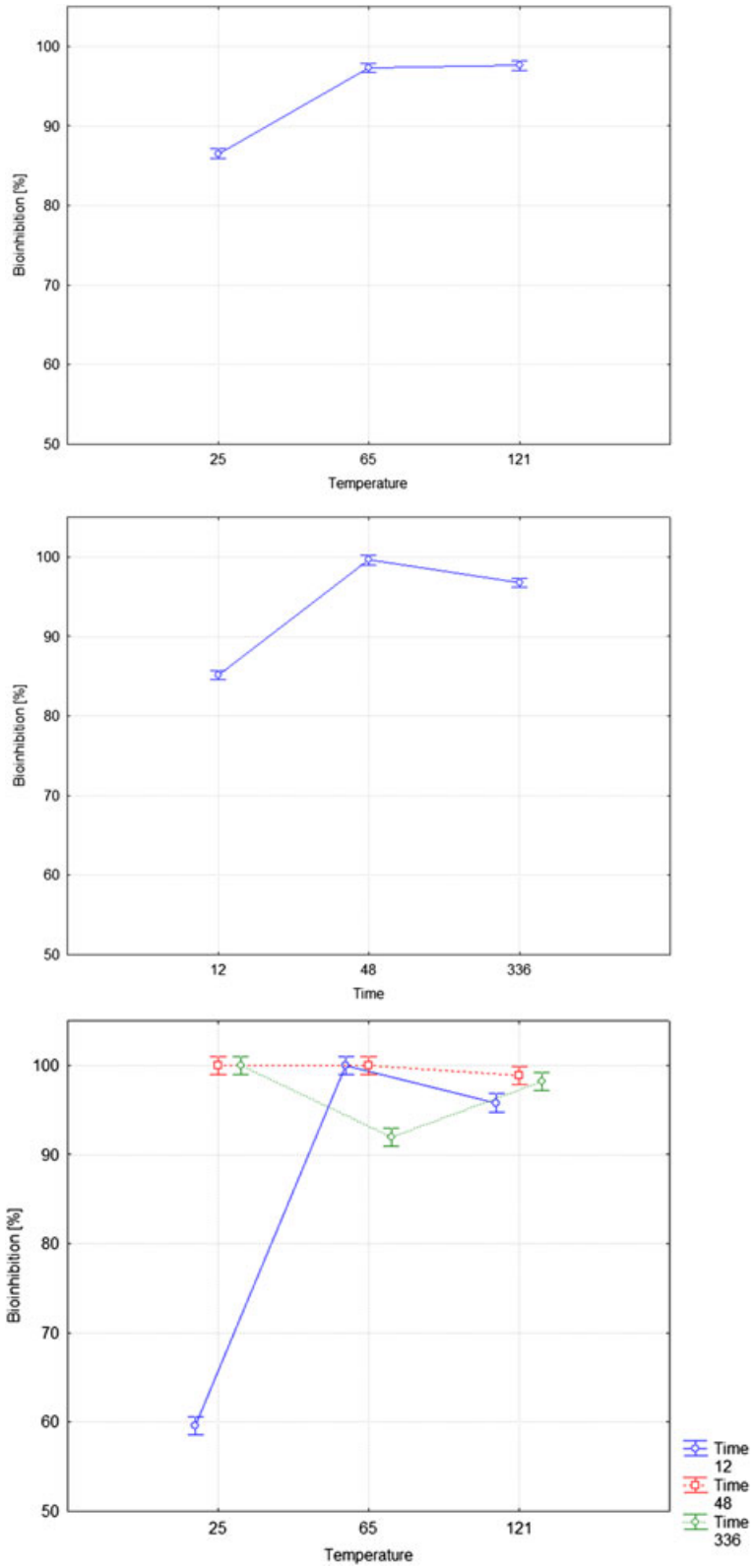

Figure 5. Effect plots for acetic acid extraction experiments: (a) temperature [°C], (b) contact time [h] and (c) interaction temperature–contact time (points are shifted aside for better perspicuity).

The three studied parameters have significant influence on acute toxicity also in DMSO extraction experiments. The temperature effect plot has maximum at 25°C and minimum at 65°C (Figure 4a). The lower inhibition of bioluminescence values at higher temperature levels could be explained by transformations of more toxic substances to less toxic products at higher temperatures. The increasing of contact time is accompanied with increase of acute toxicity (Figure 4b), which is an indication for migrations of toxic compounds during prolong migration procedures. The temperature–contact time interaction curves (Figure 4c) follow temperature effect pattern as 12 h contact time series have the lowest inhibition of bioluminescence values.

The MANOVA model for acetic acid extracts shows significance of all three effects. The increase of the temperature and contact time leads to increase of acute toxicity (Figure 5a and b). The results at higher independent variable levels are a clear indication for significant migration of toxic compounds at temperatures higher than 25°C and contact time longer than 12 h. It could be seen also on Figure 5c where bioluminescence inhibition values at 25°C and contact time 12 h for both concentration levels differ significantly.

CONCLUSIONS

The chemometric analysis of the obtained results [to assess the influence of experimental parameters (temperature, time of extraction and type of solvent) on the toxicological response of selected extraction media] has been performed. Despite its numerous advantages (such as low cost, reliability, speed and ease of screening), this approach is still not commonly used for examining food contact materials.

The results described in this study confirm that water extraction at the lowest temperature (25°C) and contact time (12 h) produces favourable conditions for the release of small molecules used as metabolic substrates by bacteria. Ethanol extraction resulted in the highest values of bioluminescence inhibition after 48 h at 65°C (indicating that toxicity increased) and showing that toxic ingredients from the packaging migrated into the extracts. Using 121°C and 336 h of extraction led to conditions favourable for bacterial growth and to decrease values indicating inhibition of bioluminescence. Studies on the toxicity of DMSO extraction present a different scenario. Prolonged contact time is accompanied by increase in acute toxicity, possibly because of migration of toxic packaging ingredients; however, increase in temperature led to a reduction in the toxicity levels of these substances.

The results of toxicity studies (where acetic acid was used as a simulant) indicate significant migration of toxic compounds with increases in time and temperature during extraction, reinforcing the hypothesis that food products with low pH values (such as those stored in cans) cause substantial damage to the resin lining inside the cans and causing increased migration of packaging material into food.

In the perspective studies, the procedures will be extended to introduce new bioassays as plausible tools in assessing toxicity/endocrine threat of food contact materials and their extracts under simulated conditions as well as an attempt will be undertaken to identify chemicals that may be responsible for elevated toxicity levels as one of steps on the way to search for novel materials that can find usage in improving packaging at least of some of the foodstuffs.

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