Assessing Acute Toxicity and Endocrine Disruption Potential of Selected Packages Internal Layers Extracts

N. Szczepańska, B. Kudłak, G. Yotova, S. Tsakovski, J. Namieśnik

***Abstract*—**The focus of the present study is migration of toxic substances from food contact materials and its actual influence on the health of the final consumer. Two food packagings (metal cans, TetraPack®) and five simulants medias (water, ethanol, acetic acid, DMSO, artificial saliva) were selected for simulation studies. For assessment of acute toxicity and endocrine disruption potential of extract samples, two biotests (Microtox® and XenoScreen YES/YAS) were performed. Multi-factor analysis of variation (MANOVA) was used to evaluate the effects of the three main factors - solvent, temperature, contact time and their interactions on the respected dependent variable (acute toxicity or estrogen disruption potential).

***Keywords*—**Biotest, extraction, food packaging, migration, toxicity.

I. Introduction

IN the scientific literature related to the widely understood issue of packaging materials designed to have contact with food (so called food contact materials), there is much information on raw materials used for their production, as well as their physiochemical properties, types and treatment parameters. However, 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. It is known that as a result of technological processes and interaction with food ingredients, migrating compounds may be subject to a transformation into various types of derivatives with physicochemical and toxicological properties different then the initial [1]. The difficulties for accurate identification and quantification of all substances released into the food, which then enter the body orally, and the lack of adequate toxicological knowledge make it impossible to assess the real danger faced by consumers. Some of the synthetic compounds used during the production of protective layers that can potentially enter the food exhibit properties similar to contaminants that are endocrine disrupting compounds [2]. The risk assessment of such bioactive compounds mixtures is complicated task since their combined action may reduce or reinforce the observed toxic effect [3]. Determination of the acute toxicity and endocrine disruption potential of the sample is only possible when one applies methods that utilize living organisms as active elements during the test [4].

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 and endocrine disruptors to foodstuffs using the acute toxicity test Microtox® and XenoScreen YES/YAS assay.

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II. Methodology

A. Experimental

Due to the fact that one of the most commonly used types of food packaging are metal cans and TetraPack® packaging they were chosen for the study. All stimulants solutions (distilled water, 5% ethanol, 3% acetic acid, 5% DMSO, artificial saliva) were prepared using reagents of analytical grade purity. Artificial saliva was prepared in accordance to guidelines described in DIN: 53160-1:2010-10 standard. The pH values of the solution were adjusted using a 1% NH_3 solution to 6.8 value. Simulation liquids were stored at +4°C prior to performing the extraction process. 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. The tests were performed using the filling method - the packages were filled with respective simulation fluids up to 5 mm below the upper edge. Since cans and TetraPack are intended for long-term storage of food, they were placed at 60°C for 10 days after filling. Additionally, in order to determine the effect of temperature on the degree of release of xenobiotics, the packagings were also exposed to 65°C and 121°C temperatures. Shaking during all experiments was performed through all the time period (orbital movement, 100 rpm).

B. Bioanalytical tests

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.

The XenoScreen YES/YAS test was used to determine hormonal potential of extracts solutions with respect to oestrogenic, antioestrogenic, androgenic and antiandrogenic

activity samples tested as the test uses genetically modified *Saccharomyces cerevisiae* with human oestrogenic and androgenic receptors (hAR). The test was performed on the basis of manufacturer's instructions with certain modifications (ref. to [5] for details). 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.

C. Statistical analysis

For each packaging three independent variables (factors) factorial design (simulant, temperature and contact time) was performed. For can and TetraPack lining extracts each of the factors temperature and contact time take three levels. This leads to 9 experiments for each food simulant as conditions for each experiment are obtained by combination of temperature and contact time levels (refer to Table 1.).

TABLE I
FACTORIAL EXPERIMENTAL DESIGN FOR INVESTIGATED PACKAGING

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

Each experiment was run in triplicate for acute toxicity and in duplicate for endocrine disruption potential determination.

Multi-factor analysis of variation (MANOVA) was used to evaluate the effects of the three main factors solvent, temperature, contact time and solvent interactions with the other factors on the respected dependent variable (acute toxicity or endocrine disruption potential). For can and TetraPack experiments five different food simulants: distilled water, ethanol, acetic acid, DMSO and artificial saliva were used. The three-way MANOVA procedure compares the acute toxicity and endocrine disruption potential results obtained at different experimental conditions using different food simulants.

III. RESULTS AND DISCUSSION

A. Microtox test results

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

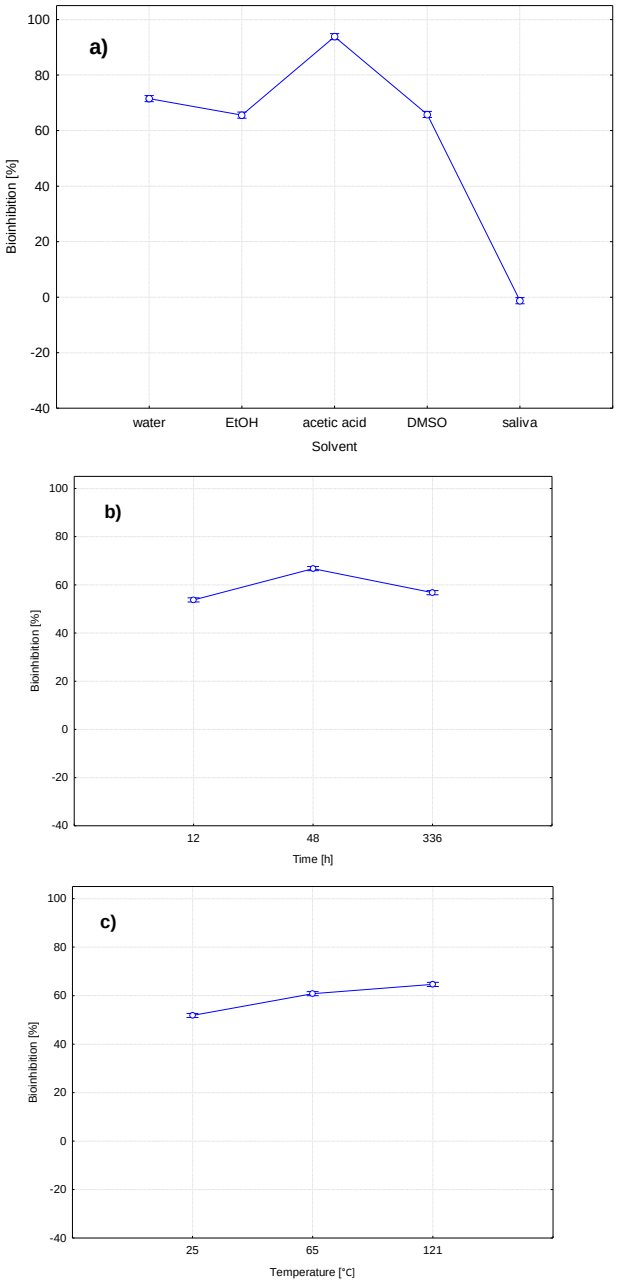

Fig. 1 Main effect plots for acute toxicity determination of can lining extracts: (a) solvents, (b) contact time and (c) temperature

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) resembles two groups of solvents. The first group of solvents consisting of water, ethanol, acetic acid and DMSO has minimum extracts acute toxicity levels at 12h and maximum at 48h following contact time effect shape presented in Fig. 1b. The toxicity of saliva extracts decreases with increasing of contact time and negative bioinhibition value at 336h (increase of bioluminescence) is an indication that hormesis occurs at the longest contract time conditions.

The solvent-temperature interaction shows quite different behavior of 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 the temperature as the effect is more pronounced for water and saliva solvents. The acute toxicity of ethanol and DMSO extracts does not possess clear relationship with

temperature. Furthermore the highest bioinhibition values of DMSO extracts are obtained at the lowest remperature (25°C).

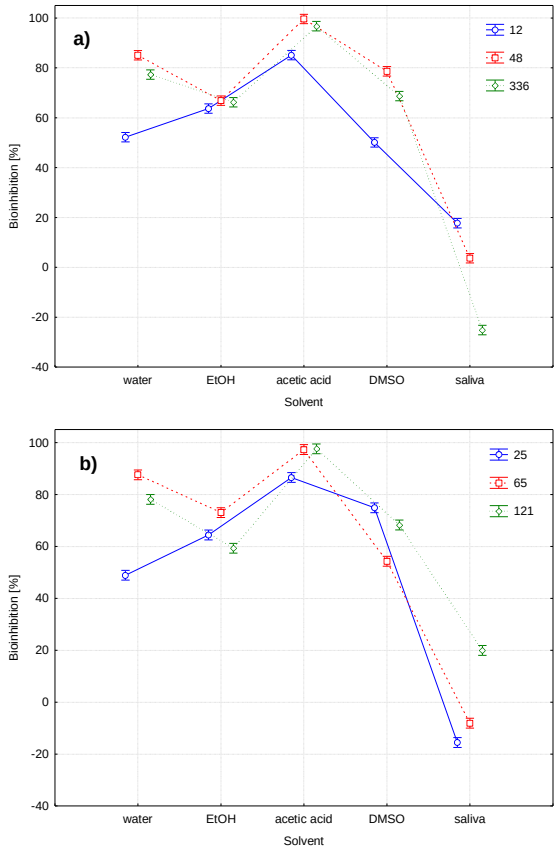

Fig. 2 Solvent interaction effect plots for acute toxicity determination of can lining extracts with: (a) contact time and (b) temperature

The MANOVA model for TetraPack lining extracts acute toxicity data shows significance of all main effects and their interactions onto acute toxicity of extracts. Again the most toxic are acetic acid extracts with bioinhibition values for all obtained extracts of 100% (Fig. 3a). Similarly like in can lining extracts experiment acetic acid is followed by less toxic water, ethanol and DMSO extracts. The toxicity of TetraPack extracts of these solvents (bioinhibition values between 20 and 45%) is significantly lower than of can lining ones. Following this trend the TetraPack saliva extracts are characterized by increase of bioluminescence with mean predicted bioinhibition value of -28.76%. The temperature and contact time do not affect strongly solvent extract acute toxicity (Fig. 3b and 3c) that is an indication that migration of toxic compounds occurs dominantly at lowest levels of both factors, namely 12h and 25°C.

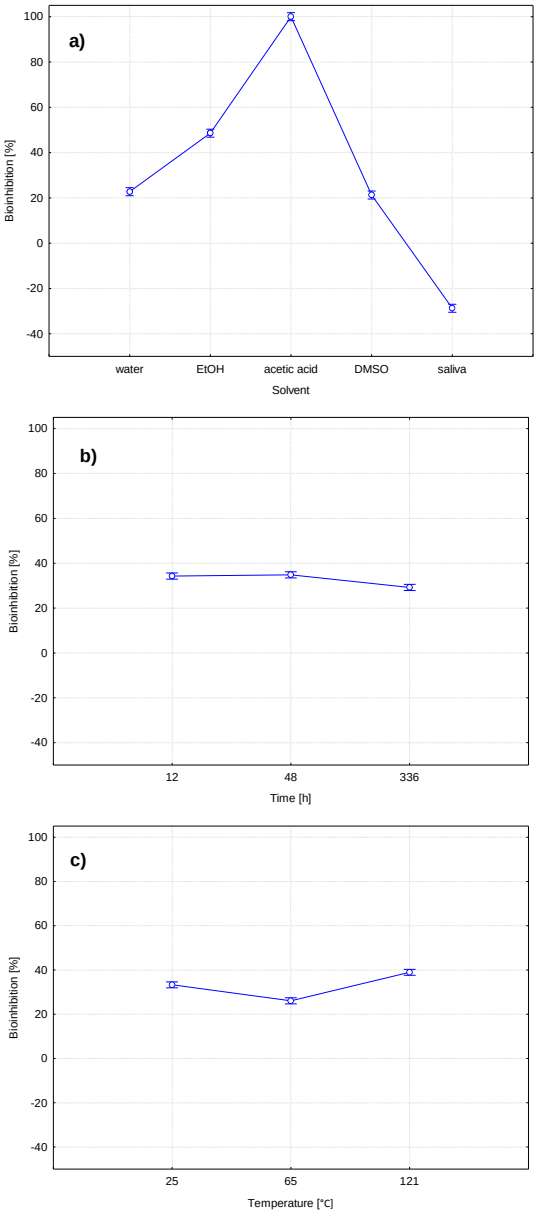

Fig. 3 Main effect plots for acute toxicity determination of TetraPack lining extracts: (a) solvents, (b) contact time and (c) temperature

The solvent-contact time and solvent-temperature interaction plots confirm small influence of extraction time and temperature on acute toxicity of particular solvent extracts (Figs. 4a and 4b). As exception in solvent-contact time plot more pronounced hormesis of saliva extracts at 336h could be pointed out (Fig. 4a). The solvent-temperature interaction plot (Fig. 4b) resembles the similar behavior of DMSO and saliva can and TetraPack extracts with maximum bioinhibition values for DMSO and saliva extracts at 25°C and 121°C respectively. In a contrary to can lining experiment the maximal acute toxicity of acetic acid TetraPack extracts is at 121°C which is an indication for additional migration of toxic compounds at the highest temperature level.

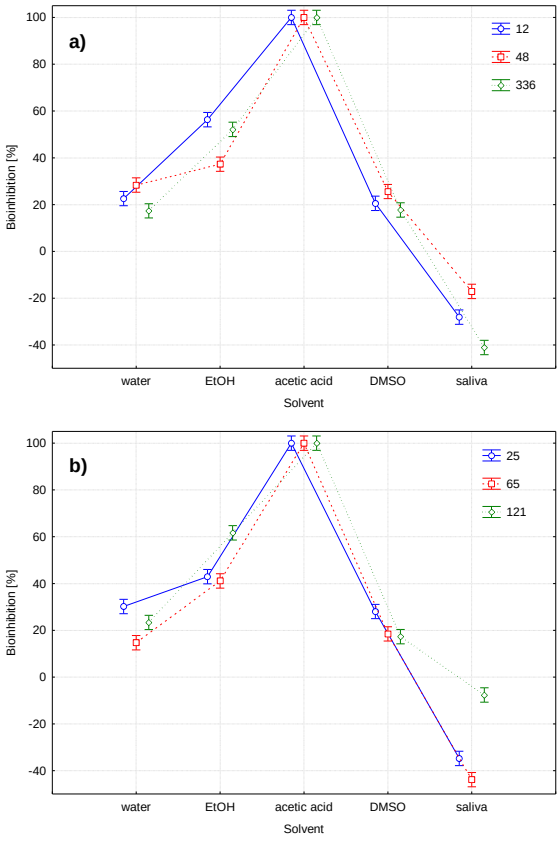

Fig. 4 Solvent interaction effect plots for acute toxicity determination of TetraPack lining extracts with: (a) contact time and (b) temperature

B. XenoScreen YES/YAS test results

MANOVA implementation to endocrine disruption potential results of can lining extracts exhibits clear difference between their estrogenic and androgenic disruption potential. The extracts of all solvents possess higher androgenic disruption potential than estrogenic one (Fig. 5). The acetic acid extracts have most significant androgenic agonistic potential (ratio higher than 1 with respect to control values) followed by DMSO and ethanol ones since water extracts possess significant androgenic antagonistic potential.

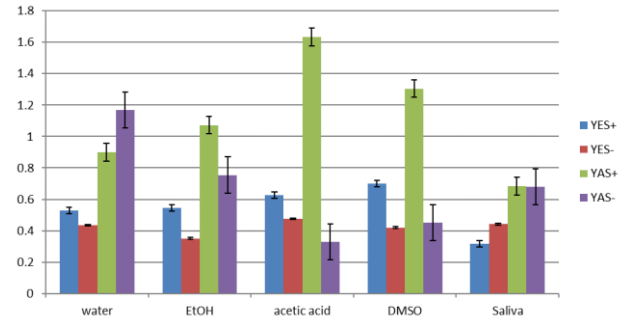

Fig. 5 Solvent effect plot for endocrine disruption potential determination of can lining extracts

The time effect plot presented on Fig. 6 confirms difference between their estrogenic and androgenic disruption potential and similarly like in can lining extracts acute toxicity study points out maximum androgenic disruption potential values at 48h.

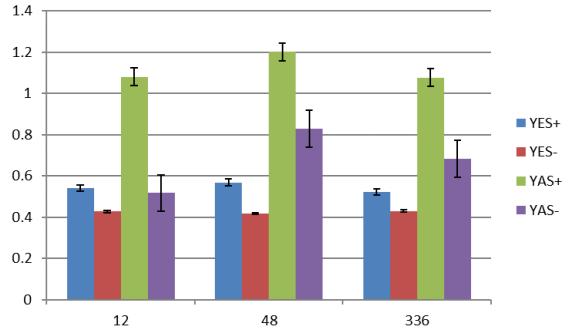

Fig. 6 Time effect plot for endocrine disruption potential determination of can lining extracts

The temperature does not affect strongly 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 migration of toxic compounds occurs dominantly at 25°C.

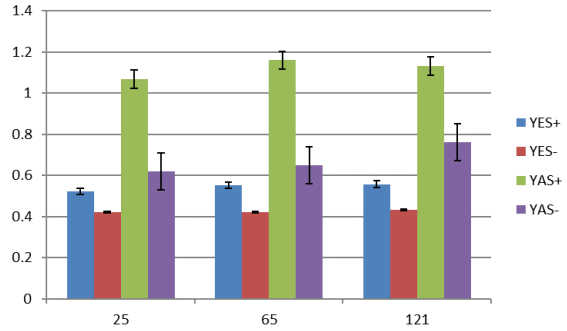

Fig. 7 Temperature effect plot for endocrine disruption potential determination of can lining extracts

The solvent-contact time interaction plot (Fig. 8) outlines the highest androgenic disruption potential for can lining extracts obtained at 48h. It should be mentioned that increasing of contact time leads to increase of androgenic disruption potential of water extracts that could be due to additional migration of toxic compounds during longer extraction procedures.

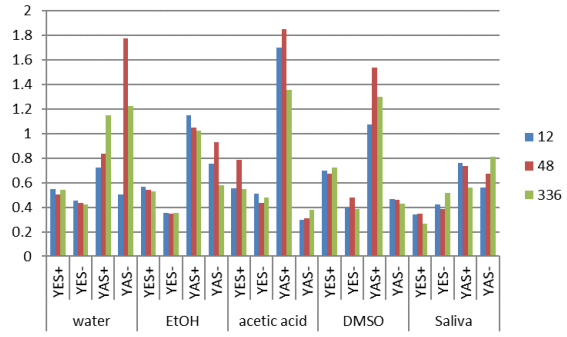

Fig. 8 Solvent-time interaction plot for endocrine disruption potential determination of can lining extracts

In general solvent-temperature interaction plot (Fig. 9) confirms the small effect of temperature on extract endocrine disruption potential. As exceptions the increase of androgenic disruption potential of water extracts and decrease of androgenic antagonistic activity of ethanol extracts could be mentioned. The water extracts exhibit the same behavior as at prolonged extractions which is a prove

that migration of compounds with higher androgenic disruption potential occur at higher temperatures and contact times. The decrease of androgenic antagonistic activity of ethanol extracts with increasing of temperature could be explained by transformations migrated substances to products with lower androgenic disruption potential.

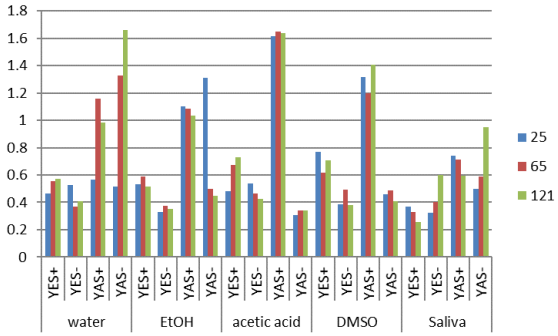

Fig. 9 Solvent-temperature interaction plot for endocrine disruption potential determination of can lining extracts

The MANOVA model for TetraPack lining extracts endocrine disruption potential shows that temperature has no significant influence on the estrogenic disruption potential of extracts and the same holds true for contact time concerning their androgenic agonistic activity. The solvent effect plot (Fig. 10) for TetraPack lining extracts outlines difference between their agonistic and antagonistic disruption potential. Extracts of all solvents possess singificantly higher estrogenic and androgenic antagonistic activity then agonistic ones. Siginficant endocrine disruption potential has acetic acid (YAS-), DMSO (YES-) and saliva (YES-) extracts.

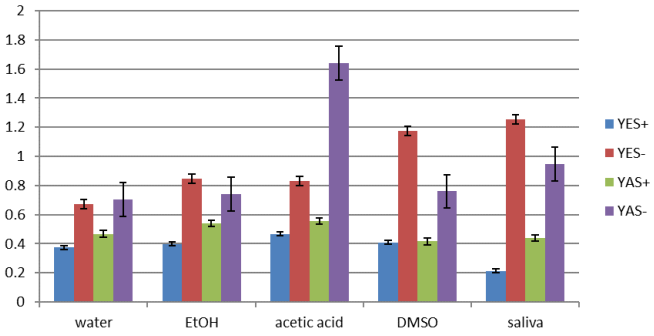

Fig. 10 Solvent effect plot for endocrine disruption potential determination of TetraPack lining extracts

The time effect does not reveal some big influence of contact time on endocrine disruption potential of stidied extracts. Only increasing of androgenic antagonistic activity with contact time increase could be excluded from this tendency.

Taking into account the MANOVA results the influnce of temperature on extract endocrine disruption potentials could be discussed only for their androgenic disruption potential. The impact of temperature on androgenic agonictic activity of extracts is not well outlined but extracts obtained at 65°C show well pronounced androgenic antagonictic maximum (Fig. 11). It could be concluded that migration of compounds with high androgenic antagonictic activity occur at 65°C and 336h contact time.

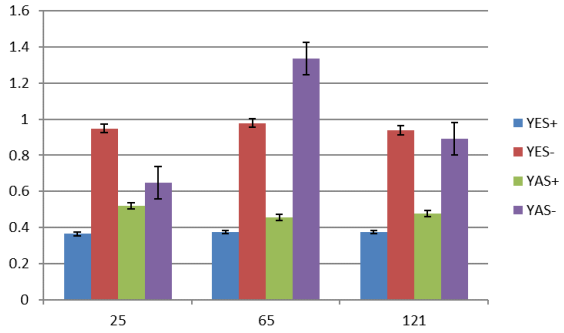

Fig. 11 Temperature effect plot for endocrine disruption potential determination of TetraPack lining extracts

The solvent-time interaction effect 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 336h supports the already commented increase with the time.

The solvent-temperature interaction plot exhibits the maximum values of antagonistic disruption potential of acetic acid, DMSO and saliva extracts obtained at 65°C (Fig. 12). It should be mentioned that both interactions show significant estrogen and androgen antagonistic potential of saliva extracts.

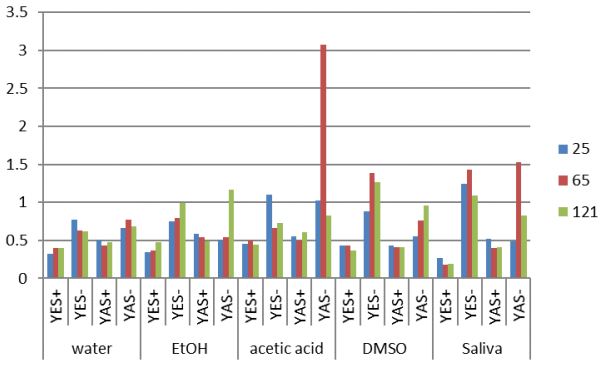

Fig. 12 Solvent-temperature interaction plot for endocrine disruption potential determination of TetraPack lining extracts

IV. Conclusions

From all stimulants studied the most toxic were can and TetraPack lining acetic acid extracts that is an indication for significant migration of toxic compounds. This migration increased with increase of contact time and temperature and justified the hypothesis that food products with low pH values cause significant damage on internal resin filling.

Can lining extracts of all simulation medias excluding distilled water and artificial saliva proved to contain androgen agonists even at 25°C and extraction time of 12h. For TetraPack extracts significant endocrine disruption potential for acetic acid, DMSO and artificial saliva were detected.

Further studies for elucidation of acute toxicity/endocrine disruption potential of extracts including chemical analysis should be performed.

Acknowledgment

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