1   **Assessment of ecotoxicity and total volatile organic compound (TVOC) emissions from**

2   **food and children's toy products**

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Graphical Abstract
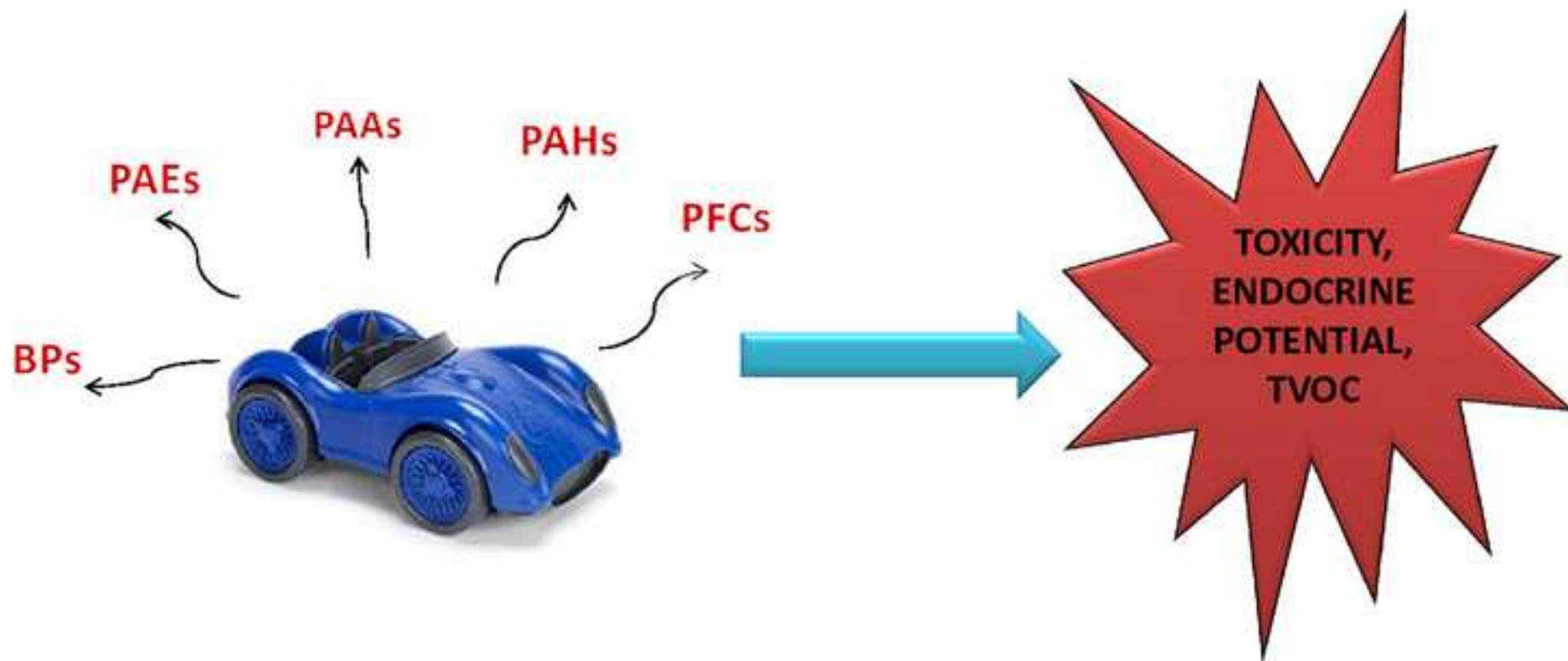
Highlights

- The assessing studies of total volatile organic compounds emissions from plastic materials were performed;
- The impact of time on toxicity of extracts of product devoted for children was investigated;
- The endocrine potential of extracts of investigated objects was <mark>assessed;</mark>
- The correlation between <mark>total volatile organic compounds</mark> emission and toxicity of children's products was estimated;
- The mathematical assessment approach was applied in the field of ecotoxicity modeling studies

14   **Abstract**

15

16   The development of new methods for identifying a broad spectrum of analytes, as well as
17   highly selective tools to provide the most accurate information regarding the processes and
18   relationships in the world, has been an area of interest for researchers for many years. The
19   information obtained with these tools provides valuable data to complement existing
20   knowledge but, above all, to identify and determine previously unknown hazards. Recently,
21   attention has been paid to the migration of xenobiotics from the surfaces of various everyday
22   objects and the resulting impacts on human health. Since children are among those most
23   vulnerable to health consequences, one of the main subjects of interest is the migration of
24   low-molecular-weight compounds from toys and products intended for children. This
25   migration has become a stimulus for research aimed at determining the degree of release of
26   compounds from popular commercially available chocolate/toy sets. One of main objectives
27   of this research was to determine the impact of time on the ecotoxicity (with *Vibrio fischeri*
28   bioluminescent bacteria) of extracts of products intended for children and to assess the
29   correlation with total volatile organic compound emissions using basic chemometric methods.
30   The studies on endocrine potential (with XenoScreen YES/YAS) of the extracts and showed
31   that compounds released from the studied objects (including packaging foils, plastic capsules
32   storing toys, most of toys studied and all chocolate samples) exhibit mostly androgenic
33   antagonistic behavior while using artificial saliva as extraction medium increased the impact
34   observed. The impact of time in most cases was positive one and increased with prolonging
35   extraction time.

36   The small-scale stationary environmental test chambers - μ-CTE™ 250 system was employed
37   to perform the studies aimed at determining the profile of total volatile organic compounds
38   (TVOCs) emissions. Due to this it was possible to state that objects from which the greatest
39   amounts of contaminants are released are plastic containers (with emission rate falling down
40   from 3273 to 2280 ng/g of material at 6 h of conditioning in elevated temperature).

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42   **Keywords**: TVOC; ecotoxicity; endocrine potential; chemometric methods; children's toys

1. Introduction

For parents around the world, the health and wellbeing of their children is an issue of paramount importance. Still, despite numerous legislative and procedural difficulties for producers of substandard toy and food producers, several accidents occurred that forced the scientific community to search for new ways to respond to this threat. Promisingly, research is being conducted to combine biological, instrumental and chemometric studies to assure holistic assessment of the quality of products intended for children and determine the impact on human well-being.

To rapidly obtain screening data about the estimated amount of volatile organic compounds (VOCs) released from the surfaces of various types of products into indoor air, research is carried out to assess the values of total volatile organic compounds (TVOCs) in a defined medium. According to published information, TVOC parameter is determine as the sum (total amount) of wide spectrum of organic compounds eluting between the defined analytical window - retention times from n-hexane to n-hexadecane on an appropriate gas chromatography capillary column (non-polar or slightly polar stationary phases) employing flame ionization detection (GC-FID) and quantified as toluene equivalents (ECA, 1997; Formela et al., 2017, 2016; Ghaffar et al., 2014; Hakkarainen, 2010; Kaykhaii and Linford, 2017; Liu et al., 2012; Massold et al., 2005). Chemical compounds such as aromatic hydrocarbons, aliphatic hydrocarbons, monoterpenes, alcohols, aldehydes and ketones (excluding formaldehyde) have a significant impact on the final value of the TVOC parameter. Depending on the object investigated, the intensity of the impact of a selected group of organic compounds on the final TVOC value varies. Preliminary determination of TVOC values allows researchers to obtain screening information on the quality of studied materials and make preliminary comparisons. In addition, the TVOC parameter consists of chemical compounds defined by the International Agency for Research on Cancer (IARC) as carcinogenic such as benzene (Group 1A), probably carcinogenic (Group 2A), or possibly carcinogenic like styrene or ethylbenzene (Group 2B) (IARC, 2016). Therefore, assessing TVOC values enables researchers to perform preliminary evaluations of the impact of studied objects on human health or the environment.

Without doubt, biological tests are becoming increasingly important in analytical practice. The main advantage of diagnostic tools is their capacity to specify the actual influence of compounds in tested samples on living organisms, taking into account all interactions between them (Wieczerzak et al., 2016). In the literature, there are many examples of studies showing

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77   that the coexistence of several pollutants leads to more severe adverse effects than predicted

78   based on the toxicity of individual components (Backhaus, 2012; Pose-Juan et al., 2016;

79   Sexton and Hattis, 2007; Silva et al., 2002). In most cases, such interactions were found in

80   compounds such as bisphenol A and related compounds, phthalates, primary aromatic amines,

81   and heavy metals (Abdul-Ghani et al., 2012; Fic et al., 2014; Michałowicz, 2014; Ramirez et

82   al., 2014; Xu et al., 2014). In a study on the individual and combined effects of bisphenol A,

83   dibutyl phthalate, and cadmium on oxidative stress and genotoxicity in HepG 2 cells,

84   synergistic interactions were noted (Li et al., 2017). In another study, a similar harmful effect

85   was observed between nonylphenol and di-N-butyl phthalate (Hu et al., 2014). These

86   compounds are plasticizers, additives and printing inks also commonly used in the production

87   of toys and other products intended for children (Lv et al., 2015; Szczepanska et al., 2016).

88   Considering these findings, it seems necessary to intensify efforts to accurately estimate the

89   degree of compound mobility and the effects of compound interactions on living organisms,

90   particularly for children who are most vulnerable to the threat. Their tendency to get to know

91   the world with their mouths in the early stages of life combined with weaker detoxicating

92   abilities makes contact with xenobiotics a potential contributing factor to serious health

93   consequences (Damstra, 2002; Mercan et al., 2015).

94   Therefore, the use of bioanalytical methods has become particularly important in research

95   aimed to estimate the degree of xenobiotic migration from object surfaces. It might be

96   observed that there is no information in the literature on the use of such a joint approach in

97   research aimed at estimating the degree of xenobiotic migration from the surface of toys to

98   liquid and gaseous phase. Therefore, the current research will make a significant contribution

99   to knowledge about the degree of compound mobility and the resulting impacts on healthy

100 children.

101 The main aim of the present study was to determine the impact of time on the ecotoxicity of

102 samples extracted from products intended for children and to evaluate the correlation with

103 TVOC emissions using basic mathematical methods performed on appropriate statistical

104 software. The ecotoxicological tests used were Microtox® and XenoScreen YES/YAS, while

105 TVOC emission rates were evaluated using a small-scale stationary emission chamber system

106 at the samples seasoning stage. The thermal desorption technique (TD) combined with gas

107 chromatography technique (GC) equipped with flame ionization detection (FID) was

108 employed at the stage of liberation and final determination of VOCs. This mathematical

109 approach is based on best-fit function modeling, with ecotoxicity or TVOC concentration as

110 dependent variable Y and time as independent variable X. As in previous studies on

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ecotoxicity modeling, the most appropriate model seems to be the polynomial fit of the $Y = a_1X^2 + a_2X + b$ type, which accurately describes the experimental data. The weight coefficient a_1 (by value and by sign) can be used to interpret the impact of time. Model validation was performed using R^2 values (multiple correlation coefficients). The research protocol presented in the following paper is an important example of interdisciplinary studies, combining aspects of analytical chemistry, environmental chemistry, ecotoxicology and mathematical/chemometric methods. All of these elements should be considered in seeking novel approaches to evaluating the quality of polymer children's toys and assessing the potential impacts on human health.

2. Materials and Methods

2.1. Objects studied

The objects studied were all elements of popular commercially available chocolate/toy sets, presented in Figure 1 for easier visualization. All elements (sealing aluminum foil package, chocolate, plastic package for toy, toy itself) of both the original product and its cheaper equivalent were subjected to both toxicological and TVOC studies in duplicate.

2.2. Extraction studies

Extraction conditions were selected to reflect plausible forms of human contact with the objects of interest. Two liquids were used as extraction media: distilled water and artificial saliva (NaCl 0.53 g/dm^3, KCl 0.33 g/dm^3, CaCl$_2$·2H$_2$O 0.15 g/dm^3, K$_2$HPO$_4$·3H$_2$O 0.76 g/dm^3, MgCl$_2$·6H$_2$O g/dm^3, K$_2$CO$_3$ 0.53 g/dm^3, 1 % HCl 0.75 g/dm^3). The experiments were run at room temperature while aliquots of extracts were sampled at 0.5, 1.0, 2.0, 6.0, and 12.0 hours. The objects were placed in glass beakers and flooded with proper extraction media (100 cm^3), sealed with Parafilm® and shaken for proper extraction times. After each period, the aliquots of extracts were sampled and subjected to toxicological studies. Prior to conducting the studies, pH was measured to verify if it was in the range of 6.5 - 7.5. In addition, the chocolate extracts were filtered to increase the clarity of the sample. Detailed information about the chemicals and reagents used in the research were enclosed in the Supplementary Material for Methodology.

2.3. Microtox® studies

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146 The acute toxicity of extracts was measured with Microtox®. The toxicity was assessed as the

147 inhibition of bioluminescence of the indicator organism (refer to Supplementary Figure 1 for

148 details). Appearance of factors that negatively influence the enzymatic activity of the

149 indicator organism inhibits the oxidation of luciferin, which is manifested as reduced

150 luminescence (Parvez et al., 2006). Because these bioluminescent organisms are highly

151 sensitive to the presence of toxic ingredients, they are used in environmental research to

152 control the degree of toxicity in water, sewage, soils, sludge, ionic liquids, and nanoparticles,

153 among others (Mortimer et al., 2008; Rossetto et al., 2014). To perform calculations it was

154 necessary to calculate the biolouminescence inhibition (BI) [%]. Q-Dixon tests were

155 performed prior to conducting respective calculation with eqs. 1-3:

156

$$R \quad \text{———} \tag{1}$$

$$\text{————} \tag{2}$$

$$\text{————} \tag{3}$$

157 *where:*

158 I_0 – initial bioluminescene of bacterial suspension,

159 I_t - bioluminescene of bacterial suspension after 30 min of exposure,

160 I_{0k} – initial bioluminescene of bacterial suspension exposed to control solution,

161 I_{tk} - bioluminescene of bacterial suspension after t = 30 min of exposure to control solution,

162 R_t – correction factor,

163 G_t – Gamma coefficient,

164 %SB – bioluminescence inhibition after time *t*.

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166 **2.4. XenoScreen YES/YAS studies**

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168 The XenoScreen YES/YAS test was used to determine hormonal potential of extracts

169 solutions with respect to oestrogenic, antioestrogenic, androgenic and antiandrogenic activity

170 samples tested as the test uses genetically modified *Saccharomyces cerevisiae* with human

171 oestrogenic and androgenic receptors (hAR) (ref. to Szczepańska et al. 2016 for details)

172   (Johnson et al., 2013; Kudłak et al., 2015; Ramirez et al., 2014). The test was performed on

173   the basis of manufacturer's instructions with certain modifications. <mark>The general diagram of</mark>

174   the XenoScreen YES/YAS procedure used for endocrine potential determinations was shown

175   on the Supplementary Figure 2.

176   Positive and solvent control were included in which experiment. Final hormone concentration

177   in positive controls ranged from 0.10 to 100 μM. The determinations were repeated three

178   times. For the oestrogenic activity, the growth factor (G) and induction ratio (IR) according to

179   the equations 4 and 5:

180    $$\overline{} \qquad\qquad\qquad\qquad\qquad (4)$$

181    $$- \ \overline{} \qquad\qquad\qquad (5)$$

182

183   where:

184   - $A_{690,S}$ and $A_{570,S}$ is absorbance of samples, respectively, at 690 nm and 570 nm;

185   - $A_{690,N}$ and $A_{570,N}$ is absorbance of the solvent control at 690 nm and 570 nm, respectively.

186   It is assumed that the tested sample has agonistic YES/YAS properties if the value of the

187   induction coefficient ≥ 1.5 (for control/calibrators solutions) and shows antagonistic

188   YES/YAS properties if the value of the induction factor ≤ 66.7 % of the value obtained for the

189   control/calibrator sample

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191   **2.5. TVOC studies and FT-IR analysis of studied products**

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193   Estimation of the amount of TVOCs released from the surfaces of studied plastic materials

194   was performed using the four small-scale stationary environmental test chambers - μ-CTE™

195   250 system <mark>(Markes International, Inc, Gold River, California, USA)</mark>. Detailed information

196   about the construction, design, operating range, working conditions and fields of application

197   of the μ-CTE™ 250 system is described in previous scientific literature (Marć et al., 2017,

198   2015; Marć and Zabiegała, 2017; Schripp et al., 2007).

199   In brief, prior to placing the samples inside the chamber, the small polymeric toys were

200   weighed. Average sample weight was 4.3 ± 1.9 g. Afterwards, selected samples of small

201   polymeric toys were placed inside each of four chambers. Then, the chamber lids were closed

202   and samples were seasoned/conditioned under static conditions (without inert gas flushing)

203   under defined conditions of temperature and time (see Supplementary Table 1). After the

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204   sample seasoning/conditioning stage, organic compounds emitted from the studied plastic
205   materials into the gaseous phase were purged from the chamber by flushing with inert gas
206   (nitrogen) at the previously defined flow rate. The samples of organic compounds were
207   collected using commercially available cylindrical containers filled with Tenax TA. The TD-
208   GC-FID system was applied to perform liberation and final determination of organic and to
209   assess the TVOC parameter values. More comprehensive information about the working
210   parameters of employed TD-GC-FID system (analytical devices working conditions) is listed
211   in Supplementary Table 1.

212   In order to obtain the general analytical information about the main type of polymer material
213   that was applied at the manufacturing process of studied brand and non-brand products (toys
214   and capsules), the Fourier transform infrared spectroscopy (FT-IR) analysis was performed.
215   The Thermo Scientific Nicolet 6700[TM] (USA) spectrometer equipped with ATR accessory
216   with diamond crystal (smart iTR) was employed, to carried out the FT-IR analysis of studied
217   samples. The measurements were conducted with 2 cm^{-1} resolution, in the range from 4000 to
218   650 cm^{-1}. The OMIC ver. 8.0 was used as the main software to analyze the obtained FT-IR
219   results. Detailed information about the FT-IR analysis of plastic materials might be found
220   elsewhere (Marć et al., 205, Formela et al., 2016, Formela at al., 2017).

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222   *2.6. Quality assurance/quality control*

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224   For quality assurance, the following parameters were set according to manufacturer's
225   guidelines: for Microtox® I_0 of bacterial suspension >70 U and $CaSO_4$ was used as positive
226   control of bacterial response (as well as $K_2Cr_2O_7$ and 3,5-dichlorophenol routinely considered
227   as positive control/reference solutions) while in case of studies with XenoScreen YES/YAS,
228   the OD_{690} of yeast cultures should be >0.3. In all cases, these requirements were fulfilled.
229   The assessed values of TVOC emissions were corrected based on the value of the blank/zero
230   sample, which was evaluated for the complete protocol under the conditions used in the
231   analysis of the selected polymeric samples. The analytes recovery from the applied sorption
232   medium - Tenax TA, was performed taking into account the studies in which a well-defined
233   amount of the internal standard solution – toluene d_8, was injected directly onto the previously
234   conditioned and purified sorbent. Next, the Tenax TA stainless steel tube with a defined mass
235   (deuterated derivative of toluene - 40 ng per tube) of standard solution was analyzed
236   employing the same analytical protocol as for the polymeric samples. Considering the
237   obtained results of analytes recovery studies, recovery values range from 96 to 104%. Making

238 an allowance for the equation of the calibration curve, the numerical values of method

239 quantification limit (MQL) of the employed analytical protocol was estimated at 0.45 ng/g

240 with toluene d_8 as a reference compound. More detailed information about the QA/QC

241 protocol might be found elsewhere (Marć et al., 2017; Marć and Zabiegała, 2017).

242 The mathematical approach is based on best-fit function modeling, with ecotoxicity or TVOC

243 concentration as dependent variable *Y* and time as independent variable *X*. As in previous

244 studies on ecotoxicity modeling, the most appropriate model seems to be the polynomial fit of

245 the '$Y = a_1X^2 + a_2X + b$' type, which accurately describes the experimental data. The weight

246 coefficient *a_1* (by value and by sign) can be used to interpret the impact of time. Model

247 validation was performed using R^2 values (multiple correlation coefficients).

248

249 **3. Results and Discussion**

250 ***3.1. Toxicological studies***

251   ***3.1.1. Impact of time on toxicity of aqueous extracts***

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253 In Table 1a, the values of a_1, a_2, b and R^2 are presented for all 10 models (refer to Figure 1, 10

254 different objects of study – parts of the original brand product, numbers in front of object

255 names denote extraction time). In almost all cases (9 out of 10 objects) *a_1* is negative, which is

256 an indication that, in general, toxicity in water decreases with time. The complex nature of the

257 toxicity/time relationship, however, is indicated by the appearance of local maxima and

258 minima in the time dependence model. Strong time dependence is observed for

259 objects/samples 1 Foil B, 2 Foil B, 6 Foil B, 12 Foil B, 1 Foil NB, and 2 Foil NB, with low

260 time dependence for 0.5 Foil B, 0.5 Foil NB, 6 Foil NB and 12 Foil NB (foil and chocolate;

261 sample 0.5 Foil NB showed very low time impact). Model adequacy is acceptable and

262 especially high for samples 1 Foil B, 2 Foil B, 6 Foil NB and 12 Foil NB. In Table 2, the

263 ecotoxic effects in water for all cases and time periods are presented. The highest toxicity is

264 observed among the "chocolate" cases, while the lowest is observed for case 1 Foil NB (the

265 strong time impact found for this case is related to the significant changes of effect from the

266 starting time to the final time).

267

268   ***3.1.2. Impact of time on toxicity of artificial saliva extracts***

269

270 In Table 1b, the values of a_1, a_2, b and R^2 are presented for all 10 models (10 different objects

271 of study) for the artificial saliva medium. For this medium, the models are less adequate. As

272 seen in Table 2, the ecotoxicity values are much lower than those in water (with the exception

273 of cases 6 Foil NB and 12 Foil NB – extracts of "chocolate" objects that correspond almost

274 entirely to the models in the water medium). All other cases (0.5 Foil B, 1 Foil B, 2 Foil B, 6

275 Foil B, 12 Foil B, 0.5 Foil NB, 1 Foil NB, 2 Foil NB) differ both in absolute value of ecotoxic

276 effects and time trends. Samples 12 Foil B and 2 Foil NB do not indicate a significant time

277 trend. Models of low adequacy are found for samples 0.5 Foil B, 1 Foil B, and 6 Foil B. For

278 the remaining cases, two (2 Foil B and 0.5 Foil NB) show a local minimum of toxicity at 2

279 hours of treatment while one (1 Foil NB) shows local maximum at 2 hours of treatment. Thus,

280 no reliable interpretations of time impacts are reasonable. However, one interesting finding is

281 the response of the "chocolate" extract samples. The toxicity data for artificial saliva extracts

282 are surprising in some extent when increasing contact time leads to increased

283 bioluminescence inhibition, it suggests that composition of simulation media used enhances

284 extractability of xenobiotics present in chocolate material making the solution more toxic

285 (impact of time is also noticeable). There are studies being performed aimed at identification

286 of substances plausibly responsible for observed elevated toxicity levels.

287

288 **_3.2. TVOC results_**

289 For 6 samples (2 Capsule B, 6 Capsule B, 12 Capsule B, 0.5 Capsule NB, 1 Capsule NB, 2

290 Capsule NB), TVOC analyses were performed. The results are interpreted using the

291 method described for Microtox effects. Polynomial models proved to be most

292 satisfactory for the results obtained. In Table 1c, the calculated parameters of the

293 polynomial model are presented. The values of TVOC emissions for all objects studied

294 and all time periods are listed in the Table 3. An attempt was made to compare both the

295 models and the experimental values for Microtox toxic effects (in water and artificial saliva)

296 and TVOC concentrations for the same objects. As presented in Table 3, there is a significant

297 difference between the emitted TVOCs for objects 2 Capsule B and 0.5 Capsule NB when

298 compared to 1 Capsule NB and 2 Capsule NB. The polynomial model satisfactorily describes

299 all samples except for 12 Capsule B, which is characterized by high TVOC concentrations

300 for very short and very long times and minima for intermediate times. Specific to these

301 models is the low value for a_1 and very high values for a_2. Thus, the a_2 value is

302 considered a better descriptor of the time trend. Therefore, for 2 Capsule B and 6

303 Capsule B samples, TVOC concentration generally decreases with time, while 12

304 Capsule B, 1 Capsule NB and 2 Capsule NB do not show significant time trends since

305 0.5 Capsule NB reveals a concentration increase with time.

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306   Taking into account the information listed in the Table 3 it was noticed a clear difference in
307   assessed TVOC parameters between original brand products and non-brand products. It was
308   observed that in both cases (small toys and containers) the original brand products were
309   characterized by much higher TVOCs emission values than studied non-brand products. To
310   explain the occurrence of this type of phenomenon, it was necessary to conduct the FT-IR
311   analysis of the brand and non-brand products. This solution gives a possibility to define the
312   main polymer material from which studied products were made. The results of FT-IT analysis
313   of brand and non-brand products were present on the Supplementary Figures from 3 to 5.
314   Considering the obtained results of FT-IR analysis of small toys and containers, both original
315   brand and non-brand products, and comparing them to the FT-IT software database, it was
316   conclude that the TVOCs emissions might be indicated by two main factors: (i) the pigment
317   application (sample color) and (ii) the type of the main polymer material which was used at
318   the studied product manufacturing process. In a first case, the polymer containers defined as
319   Capsule NB and Capsule B were mainly made of a mixture polypropylene + polyethylene-p
320   (according to performed FT-IR analysis) – in this situation, the main polymer material was the
321   same, but the color of containers was different. Due to this fact it might be pointed out that the
322   application of defined pigment might influence on the TVOCs emission values. As for the
323   second case (small toys), the higher TVOCs emissions observed for original brand products
324   might be caused by the type of polymer that was used. The original brand products (Toy 1B
325   and Toy 2B) were mainly made of acrylonitrile-butadiene-styrene (ABS) copolymer. As for
326   the non-brand products (Toy 1NB and Toy 2NB), the small toys were mainly made of
327   polyethylene (Mn 1800). The ABS copolymer is characterized by much higher TVOCs
328   emissions due to the fact, that during a manufacturing process, tree types of polymers/reagents
329   must be used to obtain the desired copolymer. Taking into consideration this information, the
330   presence of the mentioned phenomena of TVOCs emissions, might be caused by the type of
331   the polymer which was used at the manufacturing process of the brand and non-brand
332   products.

333

334   ### 3.3. XenoScreen YES/YAS results

335   Results of endocrine potential studies are presented in Table 4. In most cases, only androgenic
336   antagonistic behavior of extracts was observed, proving the objects studied to be a
337   major threat in relation to this endpoint. As other endpoints (oestrogenic agonistic,
338   oestrogenic

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339 antagonistic, and androgenic agonistic) showed no significant values or variations, no models
340 could be calculated.
341

3.4. Comparison between Microtox® ecotoxic effects and TVOC concentrations

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344 The comparison between Microtox® ecotoxic effects and TVOC concentrations was
345 performed by correlation analysis. In the first run, correlation between all water medium
346 effects with all artificial saliva effects resulted in a correlation coefficient of 0.63. This result
347 indicates that both series of effects are relatively well correlated, probably due to the very
348 high correlation between samples 6 Capsule NB and 12 Capsule NB in both series. In Table 5,
349 the correlation coefficients for the series MT water/TVOC and MT saliva/TVOC are
350 presented. Assuming that correlation coefficients (as absolute value) below 0.3 are
351 insignificant and between $0.31 - 0.60$ are of relatively low significance, one might conclude
352 that reasonable correlation (negative) is detected for cases 2 Capsule B and 12 Capsule B (MT
353 water/TVOC) and for case 6 Capsule B (positive for MT saliva/TVOC).
354

4. Conclusions

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357 The results from studies of both toxicological and instrumental analyses of TVOC
358 demonstrate that there is a significant problem with assigning the health impact of products
359 intended for children using purely instrumental methods. Although significant emissions of
360 toxicants from toys were not observed, the toxicity of chocolate extracts was significant,
361 demonstrating either its low quality or high capacity as a sink for volatile organic compounds
362 emitted from materials in contact with it. The obtained results on acute toxicity of polymer
363 material extracts coincide with data presented by other researchers. In studies where D. magna
364 was used as biologically active element, no acute toxicity was detected in water extract of PE
365 such as ABS (Lither et al., 2009, 2012). On this basis, it can be assumed that the compounds
366 released from the surface of these materials are so in low concentration that they do not cause
367 visible adverse effects in test organisms, or the exposure time was too short. It therefore
368 seems necessary to check whether this toxicity occurs after prolonged exposure (chronic
369 toxicity). As for the general law regulations about the organic compounds emission from
370 plastic materials directly into the food products, in the EU there is document defined as non-
371 legislative acts - the Commission Regulation (EU) No 10/2011 of 14 January 2011 on plastic
372 materials and articles intended to come into contact with food. In the mentioned regulation,

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373   the union list of authorised monomers, other starting substances, macromolecules obtained

374   from microbial fermentation, additives and polymer production aids was attached. Moreover,

375   mentioned document defines only a numerical values of SML [mg/kg] factor, which is the

376   specific migration limit applicable for the substance, expressed in mg substance per kg food.

377   There is a lack of general information about the limits of TVOCs emissions form plastic

378   materials which have direct contact with food products.

379   More alarming conclusions can be withdrawn from results on endocrine potential studies, as

380   the androgenic impact of extracts is clearly observable on samples extracted from plastic toys

381   and chocolate. These results may indicate that compounds such as phthalates, bisphenol A or

382   compounds from the group of brominated flame retardants are released from the surface of

383   the materials. In *in vitro* conditions, all of these compounds have been found to exhibit anti-

384   androgenic activity (Wooten et al., 2013, Kharlyngdoh et al., 2015). Moreover, many studies

385   can be found in literature confirming that it is these groups of analytes that are mainly leached

386   from the surface of children's toys (Nerev et al., 2018, Ionas et al., 2014). The presence of

387   weak anti-estrogenic responses in some samples may indicate that other xenobiotics are also

388   released from the surface of the materials. Lack of significant correlations between

389   instrumental and biological assay data may also be associated with the occurrence of synergy

390   or antagonism effect. To date, few studies have been carried out to assess the toxicity and

391   endocrine nature of compounds migrating from children's toys. Further research is needed to

392   prove the applicability of bioassay studies in assessing the impact of xenobiotics present in

393   materials intended for kids or as a validated tool in product quality assessment. Clear

394   discrepancies between different commercially available products show that such an

395   assessment is plausible, while more environmentally benign than many instrumental methods.

396   In TVOC emission profile studies, a similar characteristic of the emission profile typical of

397   the new type of plastic indoor materials was observed - a decrease in the TVOC emission rate

398   along with an increase in the storage/seasoning time inside the environmental chamber. This

399   type of relationship was observed for all studied samples of children's toys and plastic

400   containers (Marć et. al., 2015, 2017, Tirendi et. al., 2009). This may indicate that the

401   chemicals released from polymeric materials can be present in the gaseous phase between the

402   small polymeric material and the chocolate food product and might be adsorbed or dissolved

403   in the chocolate, directly affecting the human body. Additionally, significant amounts of

404   harmful organic compounds might be released directly to the gaseous phase from the surface

405   of plastic packaging, which has direct contact with the chocolate food product. However, it

406   should be clarified that further, more detailed studies are needed to define precisely the

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individual chemicals that are emitted from the surface of plastic samples directly into the gaseous phase – indoor environment. Defining the TVOC parameter or TVOC emissions profile allows researchers to obtain screening information that provides the opportunity to highlight potential problems. The future research, associated with the described interdisciplinary studies, ought to be focused on the assessing the emission rates of selected carcinogenic compounds or endocrine-disrupting chemicals that might be released from plastic materials and have direct contact and interaction with chocolate food products.

5. Acknowledgements

6. Conflict of interest

The authors declare that they have no conflict of interest.

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542

Figure Captions

Figure 1. Figure 1. Schematic representation of samples studied (legend for results description: foil B, foil NB – aluminum foil package for brand and non-brand product, respectively; capsule B, capsule NB – plastic capsule/package inside chocolate to cover toy, for brand and non-brand product, respectively; toy 1B, toy 2B,– toys inside plastic package of brand original product; toy 1NB, toy 2NB – toys inside plastic package of non-brand (fake) product; chocolate B, chocolate NB – brand (original) and non-brand (fake), respectively, chocolate sample being in contact with aluminum foil)

Supplementary Figure and Tables:

Supplementary Figure 1. Analytical procedure of Microtox® acute toxicity determination.

Supplementary Figure 2. Diagram of the procedure used in order to assess the oestrogenic and androgenic activity.

Supplementary Figure 3. The general view of FT-IR spectra of plastic container/capsules.

Supplementary Figure 4. The general view of FT-IR spectra of small polymeric toys - original brand products.

Supplementary Figure 5. The general view of FT-IR spectra of small polymeric toys – non-brand products.

Supplementary Table 1. General information about the working conditions of applied analytical equipment and techniques during the conducted TVOCs research

18

Table 1

Table 1. Values of the regression coefficients and correlations.

Extraction time [h] / object name	a_1	a_2	R^2
a)			
0.5/ Foil B	-0.14	1.55	0.1
1/ Foil B	-4.71	31.38	0.91
2/ Foil B	-4.86	33.84	0.78
6/ Foil B	-3.11	27.21	0.44
12/ Foil B	-4.72	30.18	0.52
0.5/ Foil NB	0.58	6.17	0.57
1/ Foil NB	-3.79	25.41	0.40
2/ Foil NB	-3.30	20.30	0.29
6/ Foil NB	-1.43	23.27	0.91
12/ Foil NB	-0.20	15.49	0.85
b)			
0.5/ Foil B	-4.33	29.16	0.28
1/ Foil B	1.65	-6.19	0.17
2/ Foil B	4.21	-24.63	0.48
6/ Foil B	-1.53	5.02	0.21
12/ Foil B	-0.07	-1.89	0.1 *ns*
0.5/ Foil NB	3.27	-22.34	0.68
1/ Foil NB	-0.87	1.27	0.61
2/ Foil NB	0.63	-4.49	0.1 *ns*
6/ Foil NB	-1.94	21.07	1.00
12/ Foil NB	-1.63	19.32	0.83
c)			
2/ Capsule B	499.9	-3400	0.58
6/ Capsule B	103.3	-1373	0.84
12/ Capsule B	-463.8	8415	0.1 *ns*
0,5/ Capsule NB	-679.1	5700	0.66
1/ Capsule NB	44.9	-214	0.95
2/ Capsule NB	63.9	291	0.65

Table 2

Table 2. Results of Microtox® toxicity effect in water and artificial saliva extracts

Extraction time [h] / object name	Water extraction		Artificial saliva extraction	
	Bioluminescence inhibition [%]	SD	Bioluminescence inhibition [%]	SD
0.5/ Foil B	5.862	0.023	-25.427	0.035
1/ Foil B	23.620	0.043	7.881	0.039
2/ Foil B	23.596	0.025	-20.376	0.039
6/ Foil B	15.508	0.039	15.669	0.015
12/ Foil B	14.445	0.019	-13.512	0.030
0.5/ Foil NB	13.409	0.001	-13.219	0.033
1/ Foil NB	37.354	0.010	17.876	0.018
2/ Foil NB	40.539	0.015	-11.181	0.040
6/ Foil NB	35.199	0.016	-8.220	0.013
12/ Foil NB	30.547	0.003	18.427	0.039
0.5/ Capsule B	-0.531	0.036	-22.855	0.027
1/ Capsule B	13.689	0.004	-28.576	0.014
2/ Capsule B	20.499	0.019	-49.231	0.085
6/ Capsule B	35.744	0.006	-22.879	0.112
12/ Capsule B	11.693	0.022	-22.661	0.028
0.5/ Capsule NB	2.412	0.019	-20.924	0.015
1/ Capsule NB	36.889	0.036	9.142	0.029
2/ Capsule NB	13.770	0.025	-19.689	0.029
6/ Capsule NB	40.105	0.018	-32.164	0.042
12/ Capsule NB	22.618	0.028	-20.954	0.047
0.5/ Toy 1B	-0.531	0.036	-22.855	0.027
1/ Toy 1B	2.932	0.006	-22.932	0.050
2/ Toy 1B	32.879	0.007	3.896	0.020
6/ Toy 1B	15.819	0.030	-24.171	0.042
12/ Toy 1B	32.296	0.003	-32.720	0.038
0.5/ Toy 2B	12.407	0.001	-16.125	0.136
1/ Toy 2B	7.419	0.002	-7.737	0.032
2/ Toy 2B	23.828	0.034	-14.305	0.032
6/ Toy 2B	6.921	0.021	-20.661	0.065
12/ Toy 2B	52.023	0.033	-31.369	0.041
0.5/ Toy 1NB	6.298	0.031	-18.750	0.008
1/ Toy 1NB	29.836	0.048	-15.454	0.034
2/ Toy 1NB	13.347	0.026	-16.947	0.026
6/ Toy 1NB	39.166	0.016	-34.117	0.006
12/ Toy 1NB	15.036	0.018	-29.046	0.029
0.5/ Toy 2NB	-9.460	0.026	-21.009	0.024
1/ Toy 2NB	24.147	0.013	-12.907	0.028
2/ Toy 2NB	3.728	0.031	-22.862	0.018
6/ Toy 2NB	-[1]	-	-26.985	0.100
12/ Toy 2NB	3.669	0.058	-17.358	0.036
0.5/ Chocolate B	15.912	0.056	26.540	0.056
1/ Chocolate B	19.891	0.157	42.277	0.054
2/ Chocolate B	47.140	0.012	52.922	0.011
6/ Chocolate B	68.144	0.010	61.003	0.019
12/ Chocolate B	65.233	0.022	64.473	0.019
0.5/ Chocolate NB	25.556	0.031	41.463	0.019
1/ Chocolate NB	20.311	0.096	51.217	0.011
2/ Chocolate NB	51.280	0.017	58.282	0.009
6/ Chocolate NB	72.615	0.005	83.284	0.038
12/ Chocolate NB	70.762	0.008	72.516	0.006

[1] Unrepeatable results, omitted in data treatment

Table 3

Table 3. TVOCs emission values for all cases and times studied.

object	Sample mass [g]	Sample seasoning time inside a chamber [h]	TVOC emission [ng·g^{-1}]
Capsule B	3.9902	0.5	3273.24
		1	2610.25
		2	2886.47
		6	2280.37
		10	2935.57
Capsule NB	3.8232	0.5	1114.40
		1	1123.72
		2	620.06
		6	564.16
		10	538.73
Toy 1B	6.8347	0.5	1420.44
		1	384.33
		2	440.33
		6	343.14
		10	1109.06
Toy 2B	7.3696	0.5	210.72
		1	1252.19
		2	1211.94
		6	986.86
		10	1484.63
Toy 1NB	1.8211	0.5	96.91
		1	88.05
		2	79.62
		6	100.55
		10	183.46
Toy 2NB	2.9535	0.5	415.57
		1	310.08
		2	280.00
		6	560.19
		10	545.37

Table 4

Table 4. Results of endocrine potential determinations for extracts.

Extraction time [h] / object name	YES +		YES -		YAS +		YAS -	
	water	artificial saliva	water	artificial saliva	water	artificial saliva	water	artificial saliva
0.5/ Foil B	0	0	0	1	0	0	2	2
1/ Foil B	0	0	0	0	0	0	3	0
2/ Foil B	0	0	0	0	0	0	2	5
6/ Foil B	0	0	0	2	0	0	5	4
12/ Foil B	0	0	0	0	0	0	6	3
0.5/ Foil NB	0	0	0	1	0	0	2	2
1/ Foil NB	0	0	0	0	0	0	3	1
2/ Foil NB	0	0	0	2	0	0	2	5
6/ Foil NB	0	0	0	2	0	0	5	5
12/ Foil NB	0	0	0	1	0	0	3	3
0.5/ Capsule B	0	0	1	0	0	0	2	1
1/ Capsule B	0	0	0	0	0	0	0	1
2/ Capsule B	0	0	0	0	0	0	2	3
6/ Capsule B	0	0	0	2	0	0	4	5
12/ Capsule B	0	0	1	0	2	0	6	3
0.5/ Capsule NB	0	0	0	1	2	0	1	2
1/ Capsule NB	0	0	0	0	0	0	0	1
2/ Capsule NB	0	0	0	0	0	0	2	0
6/ Capsule NB	0	0	0	0	0	0	4	3
12/ Capsule NB	0	0	0	0	2	0	6	2
0.5/ Toy 1B	0	0	1	1	0	0	2	0
1/ Toy 1B	0	0	0	0	0	0	3	2
2/ Toy 1B	0	0	0	0	0	0	2	2
6/ Toy 1B	0	0	0	0	0	0	4	5
12/ Toy 1B	0	0	0	0	0	0	3	0
0.5/ Toy 2B	0	0	1	1	0	0	2	1
1/ Toy 2B	0	0	0	0	0	0	3	2
2/ Toy 2B	0	0	0	0	0	0	2	4
6/ Toy 2B	0	0	0	0	0	0	4	5
12/ Toy 2B	0	0	0	0	0	0	2	3
0.5/ Toy 1NB	0	0	2	0	0	0	2	3
1/ Toy 1NB	0	0	0	0	0	0	3	1
2/ Toy 1NB	0	0	0	0	0	0	2	5
6/ Toy 1NB	0	0	0	0	0	0	4	0
12/ Toy 1NB	0	0	1	0	2	0	6	3
0.5/ Toy 2NB	0	0	1	0	0	0	2	2
1/ Toy 2NB	0	0	0	0	0	0	2	2
2/ Toy 2NB	0	0	0	0	0	0	2	5
6/ Toy 2NB	0	0	0	0	0	0	2	2
12/ Toy 2NB	0	0	0	0	0	0	2	2
0.5/ Chocolate B	0	0	0	0	0	0	2	3
1/ Chocolate B	0	0	0	0	0	0	2	2
2/ Chocolate B	0	0	0	0	0	0	4	5
6/ Chocolate B	0	0	0	0	0	0	5	3
12/ Chocolate B	0	0	0	0	0	0	4	3
0.5/ Chocolate NB	0	0	0	0	0	0	2	3
1/ Chocolate NB	0	0	0	0	0	0	2	2
2/ Chocolate NB	0	0	0	2	0	0	3	5
6/ Chocolate NB	0	0	0	0	0	0	5	4
12/ Chocolate NB	0	0	0	0	0	0	4	3

Explanation of numbers

0	1	2	3	4	5	6
Weak effect			**→**			**Strong effect**

Table 5

Objects	Water media	Artificial saliva
Capsule B	**-0.90**	-0.07
Capsule NB	-0.22	**0.69**
Toy 1B	**-0.88**	-0.10
Toy 2B	0.43	-0.29
Toy 1NB	-0.17	-0.54
Toy 2 2NB	-0.37	-0.37

Table 5. Correlation coefficients between bioluminescence effects for different times in relation to TVOC emissions for the same times

Objects	Water media	Artificial saliva
Capsule B	**-0.90**	-0.07
Capsule NB	-0.22	**0.69**
Toy 1B	**-0.88**	-0.10
Toy 2B	0.43	-0.29
Toy 1NB	-0.17	-0.54
Toy 2 2NB	-0.37	-0.37

Toy 1NB Toy 2NB Capsule NB Capsule B Toy 1B Toy 2B

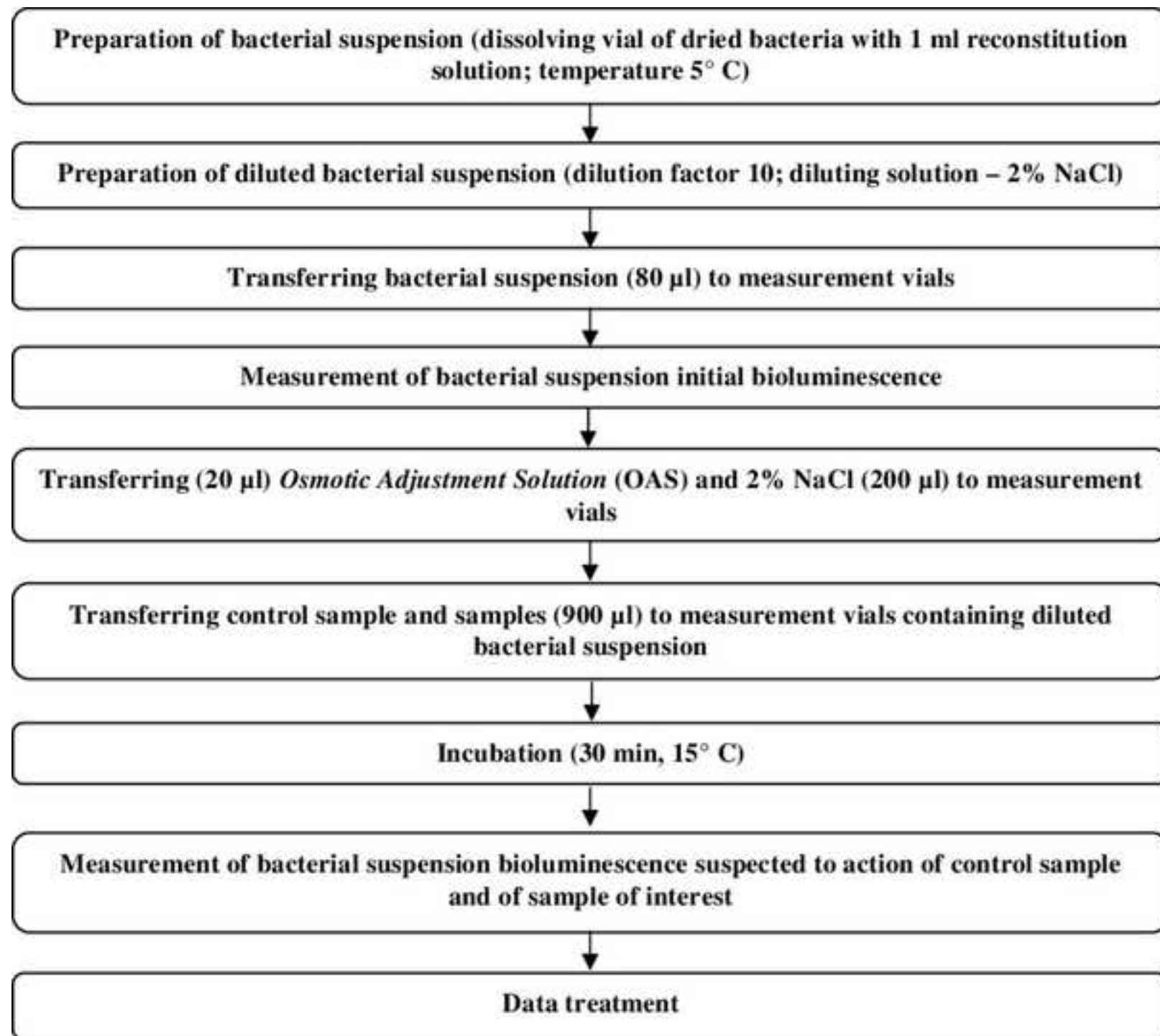Chocolate NB Foil NB Foil B Chocolate B
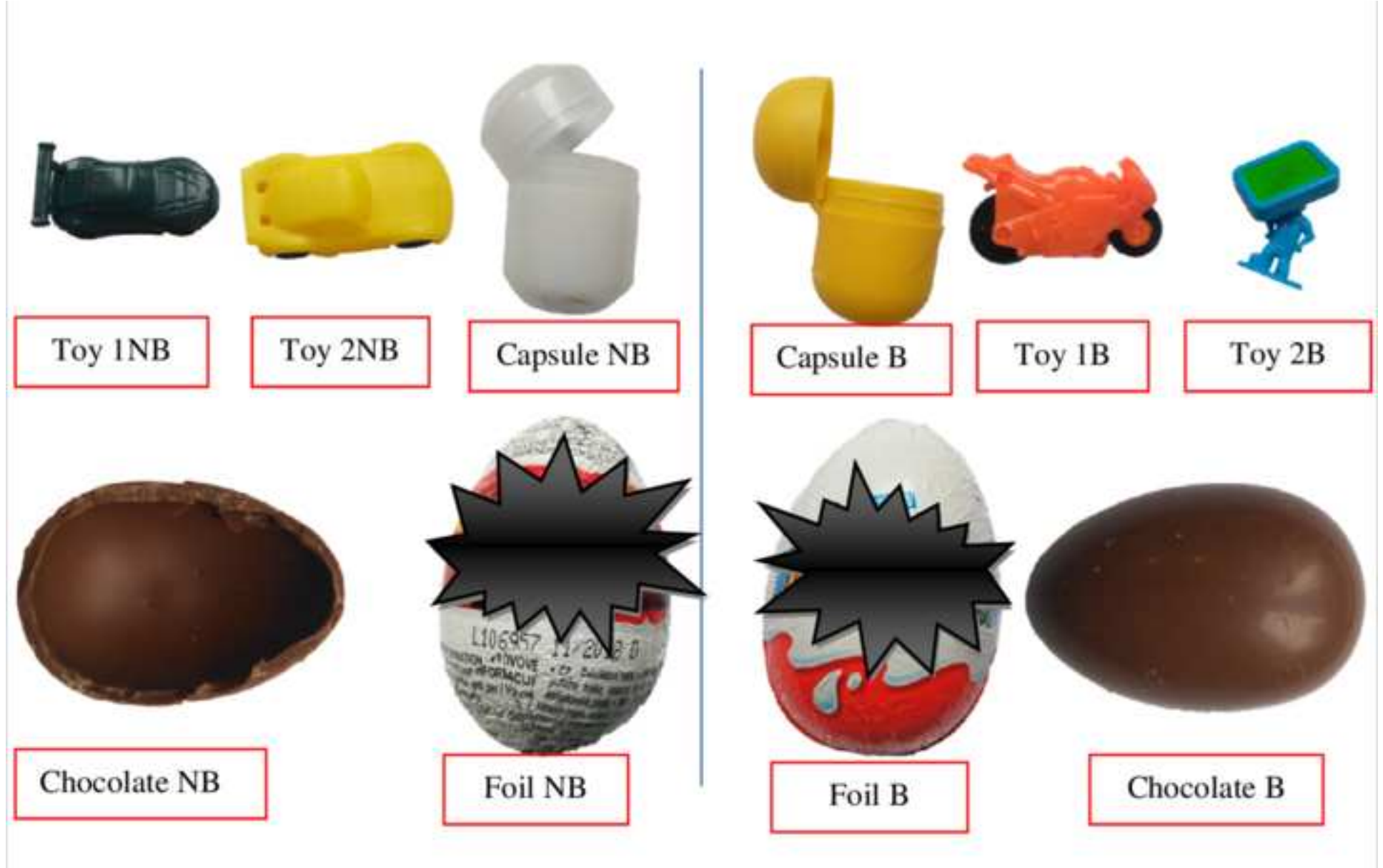
S.1. Materials and methods

S.1.1. Chemicals and reagents

Chemicals used in the research were: sodium chloride (Sigma Aldrich, Germany), dipotassium phosphate (Ciech S.A., Poland), calcium chloride (Eurochem BGD, Poland), magnesium chloride, potassium chloride, potassium carbonate, lactic acid, urea (POCH S.A., Poland), acetic acid (35-38% w/w), ammonium hydroxide (25 % w/w), (Chempur, Poland), distilled water. Microtox® 500 kit (Microtox Diluent, 2 % NaCl, lyophilized *Vibrio fischeri*, Osmotic Adjusting Solution (OAS), Microtox Acute Reagent, Reconstitution Solution (RS) was purchased from ModernWater Ltd. (GB). Ethanol (EtOH, CAS no. 64-17-5), acetic acid (CAS no. 64-19-7), dimethyl sulfoxide (DMSO, CAS no. 67-68-5), and Parafilm® were purchased from Sigma-Aldrich (Germany). All reagents were of analytical grade or higher. XenoScreen YES/YAS reagents (CPRG (chlorophenol red-β-D-galactopyranoside), basal medium, copper sulfate, L-aspartic acid, L-threonine, hAR and hER yeast cells stabilized on the filtration paper, vitamin, vials with calibrators: 5α-dihydrotestosterone, 17β-estradiol, flutamide, 4-hydroxytamoxifen) were purchased from Xenometrics G. A. (Switzerland). culture flasks with gas-permeable filter caps, gas-permeable plate sealers, 96-well plates, were purchased from GenoPlast Biochemicals (Poland). The equipment and instruments used were: Microtox® 500 (Modern Water Ltd. (GB)), electronic multi- and single-channel pipettes (Eppendorf (Germany)), CP411 pH-meter (Metron (Poland)), shaker type water bath 357 (Elpan Laboratory Instruments (Poland)).

Supplementary Table 1. General information about the working conditions of applied analytical equipment and techniques during the conducted TVOCs research

Sampling stage of analytes emitted from the surface of studied polymer products to the gaseous phase.	
Device applied to seasoning/conditioning samples of polymer products	Markes' Micro-Chamber/Thermal Extractor™ – µ-CTE™ 250 (*Markes International Inc., Gold River, California, USA*) containing four microscale chambers
Internal volume of a single chamber	114 cm^3
Seasoning/conditioning temperature	40°C
Conditions for conditioning/seasoning samples inside the chambers - procedure applied for collecting the organic compounds samples from gaseous phase	Seasoning/conditioning of polymer products (children's toys samples and plastic containers) under static conditions, without forced flow rate of inert gas through the chambers; Seasoning/conditioning at predefined time intervals: 0.5 h, 1.0 h, 2 h, 6 h, 10 h – starting from the moment when the studied samples were placed inside the chambers.
Nitrogen flow rate during the stage of collecting the organic compounds samples	50 ml/min
The elution time of the gaseous phase from the chamber to the sorption medium after a defined conditioning/seasoning time	25 min
Tool used to collect the organic compounds samples from gaseous phase	Cylindrical container filled with defined amount of sorption medium Tenax TA, 35/60 mesh (*Markes International Ltd.*) containing specify amount of internal standard solution (deuterated derivative of toluene - 40 ng per tube)
The liberation stage of analytes retained on a sorption medium (Tenax TA)	
Device employed to liberate the organic compounds from the sorption medium	Two-stage thermal desorber (*Omnisfera S.C., Gdansk, Poland*)
The 1st stage of thermal desorption heating temperature	280°C Microtrap* temperature – 0°C
The heating period of a Tenax TA tube	15 min
Helium flow rate passing through the Tenax TA tube	40 ml/min
The 2nd stage of thermal desorption heating temperature	Microtrap temperature - 300° C
Microtrap ballistic heating period	5 min
Helium flow rate passing through the microtrap in a straight line to the GC column.	2.2 mL/min
The conditions of final determination stage of analytes thermally extracted from the sorption medium	
Gas chromatography system	Hewlett-Packard 5890 GC Series II
Detector	Flame Ionisation Detector
Detector temperature	250°C
TD-GC transfer line temperature	150°C
Capillary column type	DB-1 (*J&W*) 30 m x 0.32 mm x 5 µm
Carrier gas type (flow rate)	Helium (2.2 mL/min)
Oven temperature program	40°C by 1 min; 5°C/min up to 125°C; 10°C/min up to 220°C hold 220°C by 5 min

* the microtrap tube is filled with two types of sorption medium - Carbotrap (approx. 27 mg) and Tenax TA (approx. 37 mg).

Preparation of bacterial suspension (dissolving vial of dried bacteria with 1 ml reconstitution solution; temperature 5° C)

↓

Preparation of diluted bacterial suspension (dilution factor 10; diluting solution – 2% NaCl)

↓

Transferring bacterial suspension (80 µl) to measurement vials

↓

Measurement of bacterial suspension initial bioluminescence

↓

Transferring (20 µl) *Osmotic Adjustment Solution* (OAS) and 2% NaCl (200 µl) to measurement vials

↓

Transferring control sample and samples (900 µl) to measurement vials containing diluted bacterial suspension

↓

Incubation (30 min, 15° C)

↓

Measurement of bacterial suspension bioluminescence suspected to action of control sample and of sample of interest

↓

Data treatment

Supplementary Figure 2

PREPARATION OF YEAST CULTURE
- Transfer of specially prepared filtration discs with hERα and hAR yeast cells applied into the growth medium
- Incubation (2-3 days, temp 32°C, shaking)

PREPARATION OF PLATES FOR DILUTIONS

ACTUAL SAMPLES
Transfer of 73μl of the sample into a multi-well plate

CONTROL SAMPLES
Adding 100 μl of the DMSO solution into vials containing control solutions to determine oestrogenic properties (17β-estradiol, 4-hydroxyamoxyphen) and androgenic properties (flutamide, 5α-dihydroxytestosterone)
Transfer of 73μl of the sample onto a multi-well plate

- Preparation of a series of 8 dilutions (DMSO diluting agent, dilution quotient of 3)
- Addition of 180 μl of the growth medium solution and transfer of 20 μl of prepared dilutions using a multi-channel pipette

PREPARATION OF PLATES FOR DETERMINATIONS
- Application of 20 μL of growth medium onto plates
- Application of 20 μL of selected dilutions of samples and control solutions onto plates
- Transferring 60 μL of the CPRG solution onto samples intended for determination of agonistic YES/YAS properties and 60 μL of the CPRG solution containing an appropriate amount of 17β-estradiol in the case of determination of antagonistic YES properties and 5α-dihydroxytestosterone for determination of antagonistic YAS properties.
- The addition of 100 μL of YES/YAS fungi solution to appropriate plates.

INCUBATION OF PLATES FOR DETERMINATIONS
- Protection of samples using a sealing membrane.
- Transfer of plates into a plastic container with wet tissue paper.
- Incubation (48 hours, temp 32°C, shaking)

READING PLATES FOR DETERMINATIONS
- Thorough mixing of the content of the wells using a multi-channel pipette
- Reading plates using a spectrophotometer at wavelength of 690 nm in terms of the growth of cells and wavelength of 570 nm to determine the expression of β-galactosidase