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Application of chemometric techniques in studies of toxicity of selected commercially available products for infants and children

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Abstract The goal of the present study is to assess the impact of the experimental conditions for extraction procedures (time of extraction, thermal treatment and type of extraction media) as applied to several baby and infant products checked for their possible ecotoxicological response when tested by various ecotoxicity tests (Microtox®, Ostracodtoxkit F™ and Xenoscreen YES/YAS™). The systems under consideration are multidimensional by nature and, therefore, the appropriate assessment approach was intelligent data analysis (chemometrics). Hierarchical cluster analysis (HCA) and principal component analysis (PCA) were selected as reliable data mining methods for the interpretation of the ecotoxicity data. We show that the different experimental conditions have a significant impact on the ecotoxicity levels observed, especially those measured by Microtox® and Ostracodtoxkit F™ tests. The time of contact proves to be a very significant factor for all extraction media and ecotoxicity test procedures. The present study is a pioneering effort to offer a specific expert approach for analysing links between the type of test measurement methodology and imposed experimental conditions to mimic real-life circumstances in the use of baby and infant products.

Keywords Multivariate statistics · Endocrine potential · Toxicity · Artificial human fluids · Extraction · Products for infants

Highlights • Artificial sweat and saliva were used to prepare samples for toxicological studies.
• Microtox® toxicity is reversely correlated with endocrine potential values.
• The antagonistic endocrine potential of products for infants was confirmed.
• Multivariate statistical techniques were used to analyse such elaborate dynamic systems.

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Introduction

The technological progress in the design and manufacture of a wide range of materials and also the increasing demands of customers have undoubtedly contributed to the significant development observed recently in the field of the manufacture of toys and products for children and infants. The contemporary toy market is directed towards the manufacture of toys that will positively influence child development and will in no way pose a threat to health and life (Korfali et al. 2013). Owing to their small body mass and weaker detoxification capabilities, children are especially exposed to the unfavourable influence of various factors that can disturb their proper development (Faa et al. 2012; Mercan et al. 2015). Therefore, toys and all of the products for children are subject to the

most restrictive EU regulations. The 2009/48/EC Directive contains detailed guidelines regarding safety evaluation in the scope of mechanical and physical hazards as well as the hazards connected with flammability and electricity. Considerable attention has been paid to the requirements concerning the use of chemical substances. The list of forbidden substances has been presented, including particular odorous substances that can cause allergies and also migration limits specified with reference to heavy metals and compounds classified as *Carcinogenic, Mutagenic and Reprotoxic* (CMR) compound groups. The migration limit for BPA (bisphenol A) has been established at the level of 0.1 mg/ml and 0.5 mg/kg in regards to TCEP (tris(2-chloroethyl) phosphate), TDCP (tris(1,3-dichloro-2-propyl) phosphate) and TCPP (tris(1-chloro-2-propyl) phosphate) (2009/48 EC Directive). Atypical conditions to which polymeric materials are exposed made it necessary to modify the standard methodologies used for extracting small-molecule ingredients from them. Apart from water, special liquids are also used for extraction, allowing for the reproduction of actual conditions to which objects are exposed. The most frequently used model liquids include fluids simulating the composition of bodily fluids such as artificial saliva, artificial sweat (Özer and Güçer 2012) and artificial digestive fluids (Guney Zagury 2014). The evaluation of the quality of products introduced to the market takes place based on the results of identification with the use of instrumental techniques. However, such an approach raises many doubts as small-molecule compounds released from the product are identified most often with low or even very low concentration levels (Szczepańska et al. 2016). Thus, the identification and quantitative determination of all analytes is a difficult challenge for analytical chemists, and the results obtained from instrumental analyses can often be erroneous (Ionas et al. 2016). Additionally, the weakness of such procedures consists of a significant difficulty in evaluating the results of co-existence of all chemical compounds on various levels of content and their interactions (Thomas at al. 2009). Many compounds used during the manufacture of objects made of plastic have properties similar to the compounds classified in the endocrine-disrupting compounds (EDC) group (Kudłak et al. 2015a,b; Li et al. 2010), so it is impossible to specify in detail a toxic effect evoked by a mix of xenobiotics only on the basis of its qualitative and quantitative content.

Among the tools that can be used to obtain such information are appropriate bioanalytical tests (if applied under suitable experimental conditions and design). The present study includes several levels of significance, including different categories of products for children (rubbers, teethers and nipples), different materials for their production (latex, silicone, polymers and pigments), different extraction conditions (time, temperature and varying extraction media) and various ecotoxicological tests (Microtox®, Ostracodtoxkit F™, Xenoscreen YES/YAS™) (Czech at al. 2014; Hernández-Fernández et al. 2015; Kudłak et al. 2011; Rossetto et al. 2014; Ventura et al. 2012). The bioassay battery was selected based on several criteria. Briefly, organisms from different trophic levels were selected to give a more comprehensive response to toxins plausibly present in extracts. Because XenoScreen YES/YAS is a test that is relatively new on the market, it was selected to (1) assess its sensitivity, versatility and repeatability for samples of interest; and (2) supplement toxicological information with endocrine potential information as it contains genes of human hormonal receptors. To assess the impact of given materials and experimental conditions on various organisms in such a multiparametric system, the chemometric methods of intelligent data analysis seem to be appropriate for experimental data mining. There are already numerous studies devoted to ecotoxicity testing and environmental pollution assessment using chemometrics for classification, modelling and interpretation of monitoring or laboratory experimental data, just to mention a few (Chang et al. 2012; Deljanin et al. 2016; Dubiella-Jackowska et al. 2010; López-Doval et al. 2016; Peré-Trepat et al. 2006; Platikanov et al. 2012; Wieczerzak et al. 2016). Therefore, the major goals of the present study are as follows:

- Detect similarity or dissimilarity between the test procedures,
- Detect similarity or dissimilarity between the products for infants as a production pattern,
- Detect specificity (or lack of specificity) of the production material,
- Detect discriminating factors responsible for the cluster formation within product patterns,
- Detect hidden (latent) factors describing the data set structure, and
- Assess the importance of the experimental conditions (time or temperature of extraction).

Utilizing proper chemometric tests in the treatment of multiparameter data sets allows the mining of additional

valuable information on the presence of hidden dependencies and their magnitudes. To interpret the data statistically, the hierarchical cluster analysis (HCA) and principal component analysis (PCA) were applied.

Methodology

Sample collection

Seven basic products of everyday use intended for small children and infants were assessed for the degree of release of endocrine compounds from their external surface. The research objects were nipples made of various materials (latex/silicone, including nipples marked as "BPA free"), teethers (made of different polymeric materials, also heated to mimic the disinfection process performed by parents) and colourful rubber bands used for making decorative bracelets. All products were selected from commercially available high-quality products. In recent years, more and more fears have arisen regarding the possible adverse impact of colour hand bands imported from East Asia and sold in the EU without valid CEs (European Conformity: the CE marking certifies that a product has met EU health, safety and environmental requirements, which ensure consumer safety). The research was not aimed at differentiating among products by various manufacturers but focussed on the specification of the possible threats from these products. These products and their fragments (marked with red circles, surface measured under an optical microscope with microline) were collected for research and labelled in a manner facilitating identification of the results shown in the result diagrams, which are presented in Supplementary Fig. 1.

Instruments, chemicals and reagents

Chemicals that were used for preparing simulant media were obtained from the following suppliers: 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), ammonium hydroxide (25% *w*/*w*), and acetic acid (35–38% *w*/*w*) (Chempur, Poland); and distilled water. Chemicals used for Microtox® (2% NaCl solution, lyophilized *Vibrio fischeri*, Microtox Diluent, Microtox Acute Reagent, osmotic-adjusting solution, reconstitution solution) and Ostracodtoxkit F™ (vials with algal food for chronic toxicity tests and matrix dissolving medium, Spirulina algae, six-well test plates, certified dormant eggs of *Heterocypris incongruens*) were purchased from ModernWater Ltd. (GB) and Microbiotest Inc. (Belgium), respectively. Reagents used for XenoScreen YES/YAS were purchased from Xenometrics G. A. (Switzerland). These vials contained hERα yeast cells (for the YES assay) and hAR yeast cells (for the YAS assay) on filter paper, basal medium, vitamin, L-aspartic acid, L-threonine and copper sulphate solutions, CPRG (chlorophenol red-β-D-galactopyranoside), vials with 17β-estradiol, 5α-dihydrotestosterone, 4-hydroxytamoxifen, flutamide and DMSO (dimethyl sulfoxide). Furthermore, 96-well plates, gas-permeable plate sealers and culture flasks with gas-permeable filter caps were purchased from GenoPlast Biochemicals (Poland). All reagents were of analytical grade purity or better in the case of reagents for microbiological purposes. The instruments and equipment used in the study included a Microtox® 500 of Modern Water Ltd. (GB), an Infinite® 200 microplate reader from Tecan, an incubator with a rotating platform, electronic multi- and single-channel pipettes (Eppendorf, Germany), an analytical balance from Radwag (Poland), a CP411 pH-meter from Metron (Poland) and a binocular scope from Ceti NV (Belgium).

Extraction

To assess the release of harmful ingredients from the surface of objects intended for children and infants, water and artificial sweat and saliva (to mimic in vivo realistic conditions of usage as much as possible) were used. Solutions of model liquids that were used to simulate the sweat and saliva were prepared in accordance with guidelines included in Standards DIN V 53160-2:2010-10 and DIN:53160-1:2010-10. Table 1 presents information about the quantitative composition of reagents necessary to prepare the extraction media.

The pH values of the solutions were adjusted to a value of 6.8 for the artificial saliva solution and 6.5 for artificial sweat extraction medium using a 1% NH_3 solution. The simulation liquids were stored at +4 °C until the extraction process. Products intended for children and infants were cut into small, even pieces (to obtain a piece of ca. 3 cm^2 total surface area from nipples and ca. 5 cm^2 from teethers) and placed in glass vials filled with 21 cm^3 of distilled water, artificial saliva

Table 1 List of chemicals/composition of artificial extraction media used during the studies

Simulant medium	Chemicals	Concentration [mmol/dm^3]
Artificial sweat	Sodium chloride	86
	Lactic acid	11.1
	Urea	16.65
Artificial saliva	Sodium chloride	9.07
	Calcium chloride	1.35
	Dipotassium phosphate	4.36
	Magnesium chloride	1.79
	Potassium carbonate	3.83
	Potassium chloride	4.43
	Acetic acid	16.65

or artificial sweat. In the case of decorative rubbers, the whole object (total surface area ca. 1 cm^2) was immersed in extractants. To accelerate the extraction process, vials were placed on a shaker table. After 30 min, 1 h, 2 h, 5 h and 12 h (in the case of the extraction of decorative rubbers and nipples) and 24 h, 48 h and 2 weeks (in the case of the extraction of teethers), 4 cm^3 of each sample were collected. Sampling time was chosen based on the Standard EN1186-1:2002. An additional three supplementary sampling times were added (5 h, 12 h and 2 weeks) to check how contact time affects the xenobiotic release rate. To estimate the influence of the temperature on the vials containing fragments of teethers and the effects on the size of the migration stream of the toxic ingredients, the vials were heated for 30 min at 100 °C to mimic the disinfection process conducted by parents. Two sets of tests were performed. The first group of teethers was subjected to the thermal process once on the first day of extraction, while the other group was heated three times on the first, fifth and tenth day after the beginning of the extraction process to determine whether additional exposure of the tested objects to increased temperatures has a significant influence on the intensification of the migration process. The tested samples were stored at −20 °C until the biological tests were performed.

Procedures for bioanalytical tests

Microtox® and Ostracodtoxkit F™ have been used to determine the level of toxicity occurring after a short time exposure, in addition to second test, where the effects are observed only after a longer exposure time. XenoScreen YES/YAS™ was used to determine the plausible endocrine potential of extracts studied. An adopted battery of biotests utilizing organisms at different levels of the trophic chain made it possible to check whether and in what way xenobiotics affect the organisms of varying evolutionary advancement. An additional aim of the use of environmental biotests was to check whether the results obtained are consistent with the results gathered using a test where genetically modified yeasts with human receptors were used as an active element. Detailed information on the particular bioassays and procedures used are given in the Electronic Supplementary Materials.

Quality assurance/quality control

For quality assurance of running the proper test, the following parameters according to the manufacturers' guidelines were used: for Microtox®, I_0 of bacterial suspension >70 U (chromium sulphate was used as a positive control in the bacterial stock suspension test run); for control organisms in Ostracodtoxkit F™, the mean growth increment was >400 μm while their mortality was <20%; for Xenoscreen YES/YAS, the OD_{690} of yeast cultures should be >0.3. In all cases, these requirements were fulfilled.

Methodology of multivariate statistics

In the present study, two major multivariate statistical methods were used, HCA and PCA. Both approaches are well documented in the literature and do not need detailed description (Massart and Kaufmann 1983; Vanderginste et al. 1998). In principle, HCA searches for patterns of similarity (clusters) either between objects of a certain study or between the features describing the objects. Thus, HCA is a typical unsupervised method for exploratory data analysis that makes a more reliable data interpretation possible. In the present study, the input data were normalized using a z-transform, and as similarity measures, the squared Euclidean distances were used. Further, the linkage method applied was Ward's method, and the statistical significance of the clusters obtained was tested by Sneath's criterion. The output plot was a hierarchical dendrogram.

Principal component analysis is a typical dimension reduction method. The input variables are exchanged by

new (called latent) variables or principal components, which are linear combinations of the old variables. Thus, the input data structure could be explained with lower numbers of conditional features, helpful in the data interpretation process. Mathematically, the input data matrix is decomposed into factor scores and factor loading matrices responsible for the new coordinates of the objects in the reduced feature space and the contribution of the old axes in the formation of the latent variable, respectively. In the present study, the Varimax rotation mode of PCA was applied, which helps in producing a better interpretation of the physical meaning of the latent factors.

Very frequently, HCA and PCA are carried out in parallel, and the comparison between both multivariate statistical approaches validates, to some extent, the results obtained by each method separately.

Results and discussion

Seventy-seven samples of products for children and infants (35 rubbers, 27 teethers and 15 nipples) were tested for ecotoxicity level with three different ecotoxicity tests (Microtox®, Ostracodtoxkit F™ and Xenoscreen YES/YAS™) for two media (water and artificial sweat or artificial saliva) under various extraction conditions (time and temperature). The three categories of particular products were separately chemometrically treated by hierarchical cluster analysis and principal component analysis (Varimax normalized rotation mode). All calculations were performed using the STATISTICA 8.0 software package.

Decorative rubbers

In total, there were 35 decorative rubber samples, as already mentioned, and the extraction media were water and artificial sweat. The input variables were respectively coded by the initial letters of their indication and the media of application (e.g. MTIBW stands for "Microtox® test-MT", IB stands for inhibition of bioluminescence, W stands for water, AS stands for artificial sweat or saliva (depending on object studied), OS stands for Ostracodtoxkit, M stands for mortality, GI stands for growth inhibition, YAS stands for Yeast Androgen Assay, YES stands for Yeast Oestrogen Assay, A stands for agonist and AN stands for antagonist).

HCA and PCA for decorative rubbers (patterns of similarity between ecotoxicity tests)

In Fig. 1 a, the hierarchical dendrogram for clustering of all 14 variables is shown. The following four significant clusters are formed:

K1: *YASANAS*, *YASANW*, *YASAGAS*, *YESANW*, *YASAGW*, *YESANAS*, *YESAGAS* and *YESAGW*;
K2: *OGIW* and *OMAS*;
K3: *OGIAS* and *OMW*; and
K4: *MTIBAS* and *MTIBW*.

As shown in Fig. 1 a, there is a clear distinction between the three different ecotoxicity tests. Xenoscreen YES/YAS™ tests for both water and artificial sweat are very homogeneous and indicate very similar results not only for the media tested but also for the extraction time conditions. Therefore, in principle, each one of the tests should deliver comparable levels of toxicity (quite low ones) for different extraction times and various media. No differentiation with respect to the tested objects and extraction conditions was stated.

The Ostracodtoxkit F™ method is more specific. Surprisingly, there is a correlation between data for growth inhibition values (in water media) and mortality (in artificial sweat) and between growth inhibition values in artificial sweat and mortality in water. This ecotoxicity test could therefore be used to distinguish between media and could check the impact of the media in the extraction process. Since there is a lack of correlation between data for the two modes of determination (growth inhibition and mortality), there is an option to select one of them in determining ecotoxicity in different media.

For the Microtox® test, a relatively good correlation was found for data of both media, and the data appear not to be specific for the tested media.

PCA confirms conclusions stated by the HCA. Supplementary Table 1 provides the factor loadings of three identified latent factors responsible for the data set structure. The results of HCA are simply repeated, and the separation between the tests is even more reasonable. All Xenoscreen YES/YAS™ tests are included in PC1 with high factor loadings, and negatively correlated Microtox® tests for the different media and negatively correlated Ostracodtoxkit F™ tests (growth inhibition as endpoint, both media) are included in PC2. In PC3, one

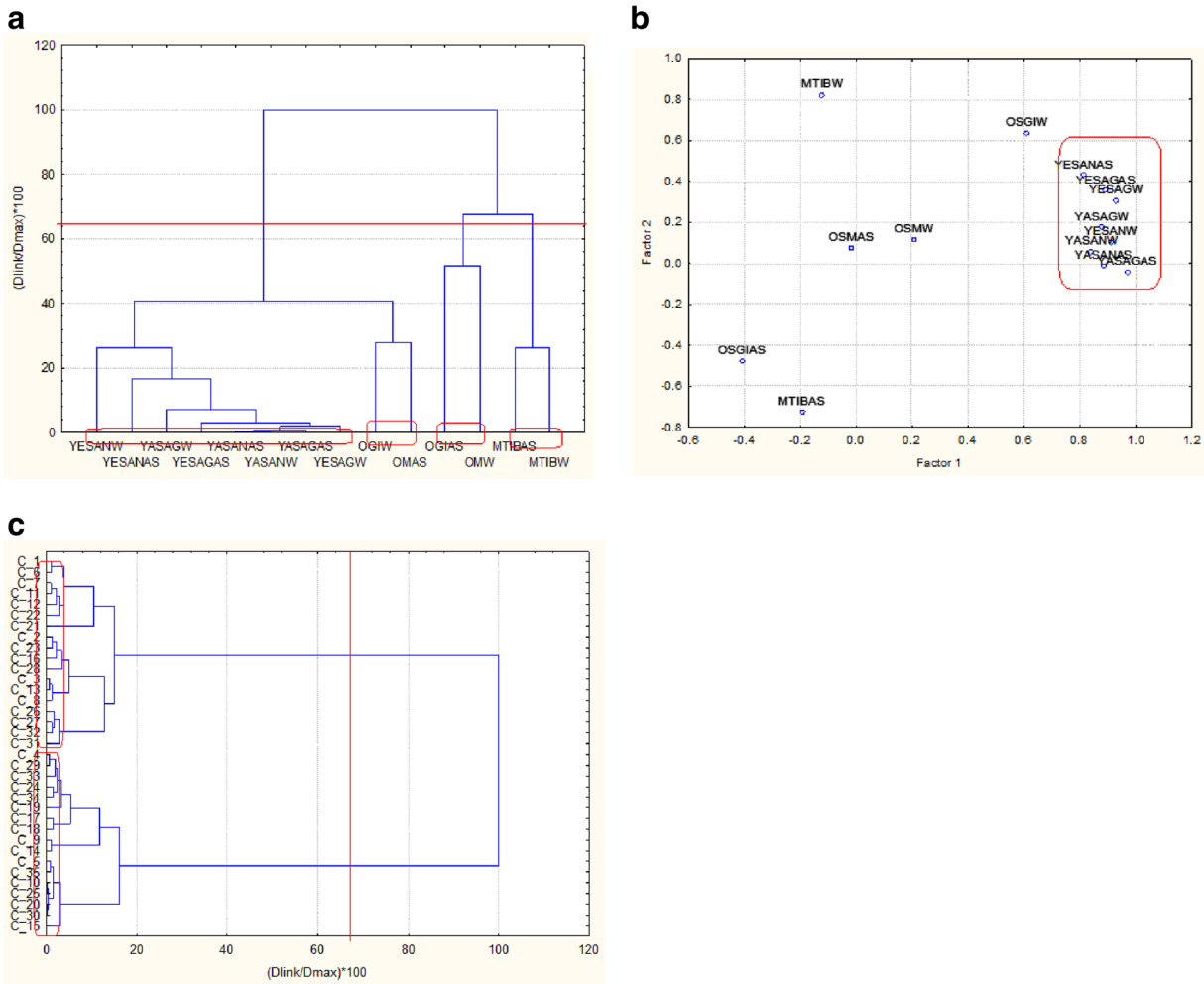

Fig. 1 Graphical representation of **a** hierarchical dendrogram for clustering of ecotoxicity tests; **b** PC1 vs. PC2 biplot (factor loadings); **c** hierarchical dendrogram for 35 decorative rubbers as objects

observes the negative correlation for the mortality mode of Ostracodtoxkit F™ for both media. Therefore, the chemometric analysis reveals that for the Microtox® and Ostracodtoxkit F™ test kits, the media (water or artificial sweat) are a significant factor since for the Xenoscreen YES/YAS™ test, the media (water or artificial sweat) are not. The loadings in bold are statistically significant. This observation is demonstrated once more in the next plot (Fig. 1 b).

The compact group of Xenoscreen YES/YAS™ tests is well documented as well as the reverse correlation for the three other coupled tests. At the same time, it could be accepted that one could define a positive correlation between OSMAS and OSMW or between OSGIAS and MTIBAS. Most likely, the most severe affecting factor is not the media or the test but the extraction conditions, a factor that needs additional attention. In any case, the responses obtained by the Xenoscreen YES/YAS™ test differ from the responses achieved by the other two tests.

HCA of decorative rubbers (patterns of objects)

The clustering of the objects (Fig. 1 c, Supplementary Table 7) indicates the formation of two major clusters. One of the clusters (the upper part of the plot, left) consists of 18 objects (K2), and the other consists of 17 objects (K1). It could immediately be determined that K1 includes objects treated for a longer time (>2 h), whereas K2 is related to shorter extraction times.

K1: *1*, *6*, *7*, *11*, *12*, *22*, *21*, *2*, *23*, *16*, *28*, *3*, *13*, *8*, *26*, *27*, *32* and *31*, whereas

K2: *4*, *29*, *33*, *24*, *34*, *19*, *17*, *18*, *9*, *14*, *5*, *35*, *10*, *25*, *20*, *30* and *15*.

In Supplementary Table 2 (supplementary materials), the average values of the toxicity determined by various tests for each of the identified clusters are presented (M1 and M2—two modes of Microtox®; Os1, Os2, Os3 and Os4—four modes for Ostracodtoxkit F™; and YY1–YY8—eight modes of the Xenoscreen YES/YAS™ test).

Obviously, k1r (upper row in the Supplementary Table 2) is characterized by a lower level of toxicity (for longer extraction times), except for the Microtox® test and k2r (lower row of the table), which have slightly higher levels of toxicity (for shorter extraction times). Thus, the role of time of extraction is clarified: longer extraction is related to lower toxicity levels.

Teethers

The number of objects for this category of products is 27 in total. The number of features is the same (14), but instead of artificial sweat, the ecotoxicity is determined in another medium, artificial saliva. The goals of the multivariate analysis are the same as for decorative rubbers.

HCA and PCA for teethers (patterns of similarity between ecotoxicity tests)

In Fig. 2 a, the hierarchical dendrogram for the linkage of ecotoxicity tests for teethers is shown. The result (refer to Fig. 2 a) varies slightly compared to the situation with products made of rubber. The Xenoscreen YES/YAS™ test is still homogeneous, but in three situations, these modifications correlate with Ostracodtoxkit F™ (clusters YASAGW, OGIW and OMW) for determinations in water or clusters (YASANASL and YASANW) linked to the outlier for the first level of Sneath's cluster significance criterion MIBW. Ostracodtoxkit F™ is relatively homogeneous for teethers (all modes in one cluster for the second level of cluster significance). For the Microtox® test, the extraction medium is a significant factor but, in general, it is linked to Xenoscreen YES/YAS™ studies. No distinct separation between the extraction media is achieved by the application of any of the ecotoxicity tests. Each test indicates the real toxicity values in each medium.

In Supplementary Table 3 (supplementary materials), the factor loadings are presented. PC1 indicates (37.56% explanation of the total variance) the relative homogeneity of the Xenoscreen YES/YAS™ test excluding, however, YASANW and YASANASL, which are well correlated but form an independent factor PC2 (20.56% of the total variance). In PC1, we detected the reverse correlation for the two extraction media analysed by Microtox® but determined that there is agreement with many of the Xenoscreen YES/YAS™ modes. Water and artificial saliva media are well separated by Ostracodtoxkit F™ test as observed in PC3 and PC4.

HCA of teethers (patterns of objects)

In the next step, the 27 teether cases were subjected to HCA. Figure 2 b shows the hierarchical dendrogram for

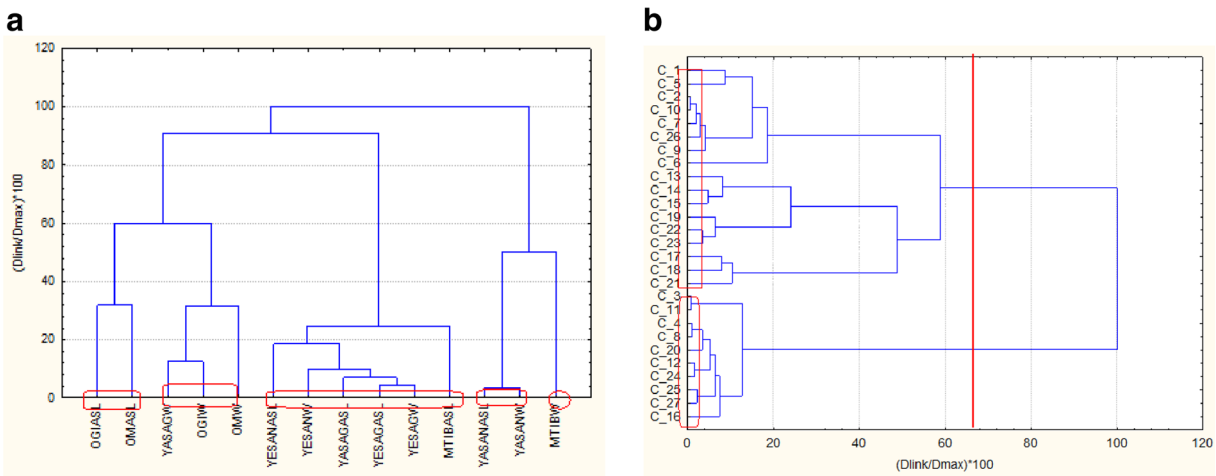

Fig. 2 Graphical representation of **a** hierarchical dendrogram for ecotoxicity tests; **b** hierarchical dendrogram for 27 teethers' extracts

the objects (refer to Supplementary Table 8 for objects decoding).

Two clusters are formed for the first level of cluster significance, as follows:

K1: *3*, *11*, *4*, *8*, *20*, *12*, *24*, *25*, *27* and *16* and
K2: *17*, *18*, *21*, *13*, *14*, *15*, *19*, *22*, *23*, *1*, *5*, *2*, *10*, *7*, *26*, *9*, *6*.

Cases 1 to 12 are treated without heating, and the others are treated after thermal treatment. The separation does not indicate any relationship with the temperature and duration of thermal treatment. The first four rows in the Supplementary Table 4 show the average values of each test for each of the four clusters (first level of significance), and the fifth row is the average value for the second cluster if the second level of cluster significance is used.

We could conclude that the teethers are separated because of the lowest toxicity indicated by Microtox® in water and relatively higher toxicity in artificial saliva. The YAS test indicated an antagonistic relationship; water or artificial saliva test results are different from the other Xenoscreen YES/YAS™ results (indication of higher endocrine potential). No other specificity is evident.

Nipples

For the third type of infant products, 15 objects were treated by the same ecotoxicity test and for the same extraction media (water and artificial saliva). The extraction is performed for different times but without heating.

HCA and PCA for nipples (patterns of similarity between ecotoxicity tests)

In Fig. 3 a, the hierarchical dendrogram for linkage between ecotoxicity tests as applied to nipple products is shown. The following two clusters are formed:

K1: OGIASL, OMASL and MTIBASL and
K2: OMW, YESANASL, YESANW, YASAGASL, YESAGASL, YASAGW, YASANASL, YASANW, YESAGW, OGIW and MTIBW.

As in the cases of previous materials (rubbers and teethers), the homogeneity of the Xenoscreen YES/YAS™ tests is proven since all tests belong to one and the same cluster. In the same large cluster, there is a correlation between the two Ostracodtoxkit F™ test endpoints in water media along with a Microtox® test for the same media, namely for OMW, OGIW and MTIBW.

In K1, the similarity between Ostracodtoxkit F™ and Microtox® tests in artificial saliva is indicated. Therefore, for the nipple group of products, a specificity of the extraction media when applying Ostracodtoxkit F™ and Microtox® tests could be found. These results are confirmed by PCA.

Two latent factors explain nearly 80% of the total variance and are responsible for the data set structure.

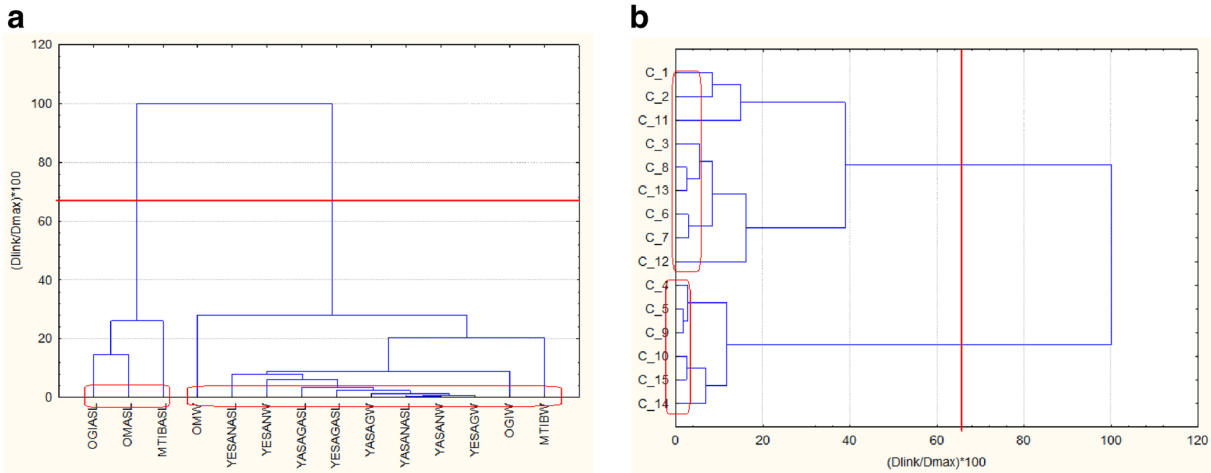

Fig. 3 Graphical representation of results of **a** hierarchical dendrogram for ecotoxicity tests and **b** hierarchical dendrogram for 15 nipples' extracts cases

They indicate the resemblance to HCA by the statistically significant factor loadings for each principal component.

HCA of nipples (patterns of objects)

In the next dendrogram (Fig. 3 b, Supplementary Table 9), the hierarchical dendrogram for the linkage among the 15 nipples objects is shown.

The following two clusters are formed:

K1: K1: 1, 2, 11, 3, 8, 13, 6, 7, 12 and
K2: K2: 4, 5, 9, 10, 15, 14.

In general, a relatively good separation with respect to extraction time is achieved. K2 includes cases subjected to longer extraction time (5 and 12 h), and the K1 indicates the similarity between cases subject to shorter extraction time (0.5 to 2 h).

Another discrimination between clusters could be achieved (with respect to ecotoxicity values offered by the different tests) if the average values of each test and for each cluster are calculated.

At shorter extraction times (cluster k1n, refer to Supplementary Table 6), the ecotoxicity in the water medium is higher compared to the ecotoxicity found for water extraction for longer periods (cluster k2n). For Microtox® and Ostracodtoxkit F™ tests, if the extraction medium is artificial saliva, a short time extraction leads to lower toxicity compared to longer extraction times.

In case of the Xenoscreen test, extraction in water and artificial saliva media delivers higher ecotoxicity responses for shorter extraction times, an indication of the role of the ecotoxicological tests applied and the extraction time.

Impact of the extraction conditions

The assessment of the extraction conditions is a substantial component of the study. These conditions are explicitly included in the discussions above, but in this section, an effort is made to summarize the extraction time impact and thermal treatment and material impacts. The relationship between extraction conditions and ecotoxicity tests has to be analysed to suggest proper conditions for application of the different tests.

Extraction time impact

In Fig. 4, the ecotoxicity responses for the two patterns (identified as long time and short time extraction clusters) for each of the baby and infant products (rubbers, teethers and nipples) are presented. All responses for the Xenoscreen YES/YAS™ test (points 7 to 14 on the plot) are observed to be very homogeneous (as already shown by cluster analysis) and indicate a constant low level of ecotoxicity independent of extraction time for all tested materials.

The other two tests show much more dynamic behaviour depending on extraction time. For short extraction times for all products, a negative toxicity is determined by the Microtox® test for artificial sweat extracts (point 2 on the plot) for all products (the dashed lines on the figure). The Ostracodtoxkit F™ test indicates relatively higher levels of toxicity for the same time of extraction, although they do not reach values much higher than 0 (points 3 to 6 on the plot). Below, the

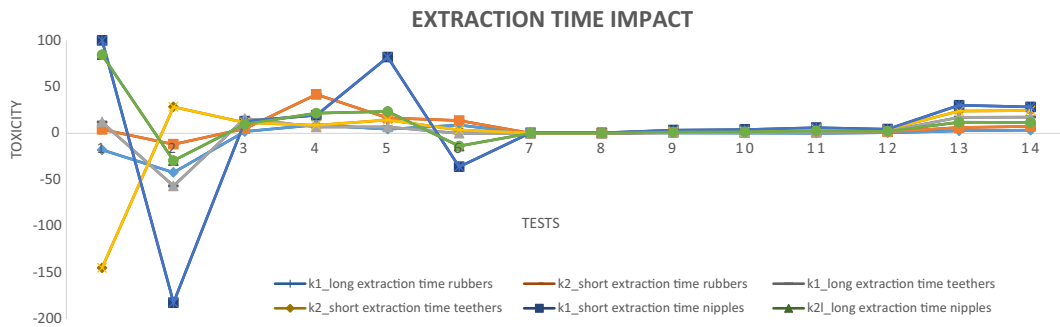

Fig. 4 Ecotoxicity for different extraction times for each one of the clusters identified (rubbers, teethers, nipples) for the various ecotoxicity tests (notations: *1*, *2*—MICROTOX; *3*, *4*, *5*, *6*—Ostracodtoxkit F™; *7*, *8*, *9*, *10*—Xenoscreen YES; *11*, *12*, *13*, *14*—Xenoscreen YAS)

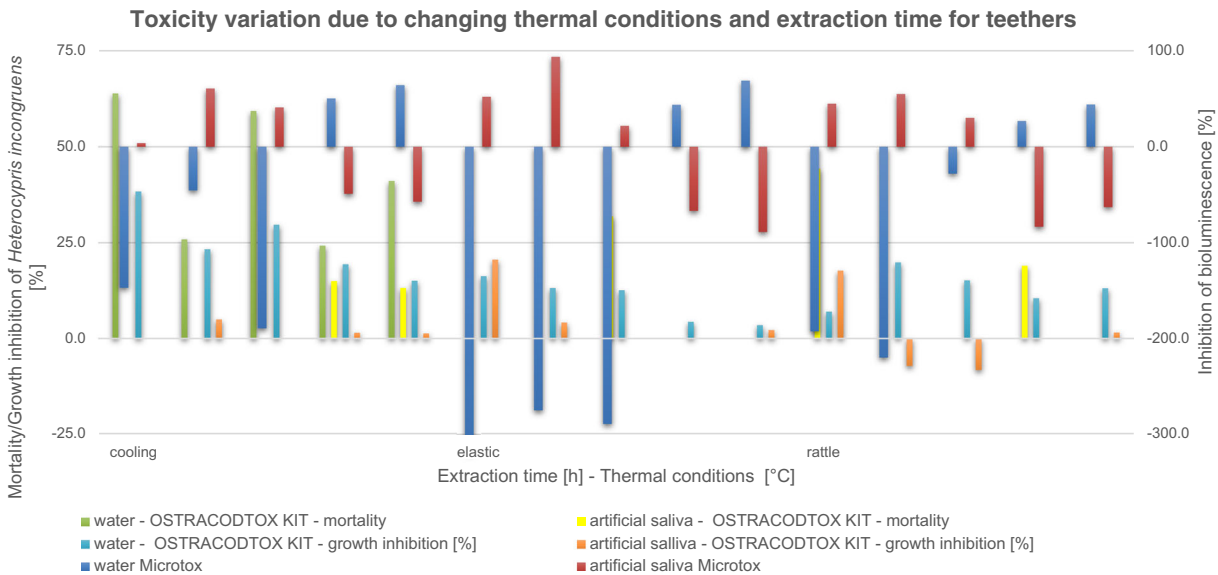

Fig. 5 The impact of thermal treatment and extraction time of teethers products on ecotoxicity (Microtox® and Ostracodtoxkit F™ tests)

product discrimination is shown (maximum level of toxicity for nipples at short time extraction in artificial saliva).

For extraction using long times, relatively higher levels of toxicity are registered for teethers (by Microtox®) and for rubbers (by Ostracodtoxkit F™). Again, a minor discrimination effect by products and tests is observed for prolonged extraction time.

Maximal toxicity is found by the Microtox® test for nipple product both for short and long time extraction in water and by Ostracodtoxkit F™ for short time extraction of nipples in artificial saliva.

Thermal treatment and material impact

In Fig. 5, an effort is made to assess the impact of thermal treatment and extraction time of teether products on ecotoxicity (Microtox® and Ostracodtoxkit F™ tests). Once again, in the case of the Xenoscreen YES/YAS™ test, homogeneity with respect to the registered responses of ecotoxicity is observed (generally low level of ecotoxicity), and the test is not thoroughly discussed.

If the Microtox® test is applied on aqueous extracts, the heating diminishes the ecotoxicity levels in general. An increase is observed for the double heating procedure. In the artificial saliva medium, a reverse trend is observed: prolonged heating diminishes the ecotoxicity levels. The elastic material showed the lowest toxicity.

The Ostracodtoxkit F™ test showed a general decrease in the toxicity levels of aqueous extracts with the increase in time of the thermal treatment of cooling materials. In the artificial saliva medium, an increase of the ecotoxicity for elastic materials is registered. Prolonged thermal treatment decreases the toxicity level.

Conclusions

The present study addresses the evaluation of a complicated data set where a variety of experimental conditions (extraction medium, extraction time, thermal treatment and three categories of baby products) is evaluated with different ecotoxicity tests. An effort was made to assess the different conditions and to reveal relationships among different objects and features of interest. The intelligent data analyses applied (hierarchical cluster analysis and principal component analysis) have indicated that the impact of the various experimental conditions is significant and, often, multidirectional.

One of the most significant conclusions in the present study is the proven difference in ecotoxicological response reached by the Xenoscreen YES/YAS™ test compared to the ecotoxicological responses from the Microtox® and Ostracodtoxkit F™ tests. This homogeneity holds true regardless of the various extraction conditions, extraction media and product materials. In

Table 2 Comparison of ecotoxicity levels for Microtox®, Ostracodtoxkit F™ and Xenoscreen YES/YAS tests for different usage conditions

Contact time	Product	Microtox®		Ostracodtoxkit F™				Xenoscreen YES/YAS							
		Bioluminescence inhibition [%]		Mortality [%]		Growth inhibition [%]		YES(+)		YES(−)		YAS(+)		YAS(−)	
		Water	Artificial sweat or saliva	Water	Artificial sweat or saliva	Water	Artificial sweat or saliva	Water	Artificial sweat or saliva	Water	Artificial sweat or saliva	Water	Artificial sweat or saliva	Water	Artificial sweat or saliva
Long	Decorative rubbers	N	N	Y	Y	Y	Y	N	N	Y	Y	N	N	Y	Y
Short	Decorative rubbers	N	N	Y	Y	Y	Y	N	N	Y	Y	N	N	Y	Y
Long	Teethers	Y	N	Y	Y	Y	N	N	N	N	N	N	N	Y	Y
Short	Teethers	N	Y	Y	Y	Y	Y	N	N	Y	Y	N	N	Y	Y
Long	Nipples	Y	N	Y	Y	Y	N	N	N	Y	Y	N	N	Y	Y
Short	Nipples	Y	N	Y	Y	Y	N	N	N	Y	Y	N	N	Y	Y

Y means positive values of ecotoxicity/endocrine potential; N means negative values of ecotoxicity/endocrine potential under given test condition

almost all cases, this test indicates relatively low positive ecotoxicity levels.

The application of multivariate statistical analysis has shown that in all clustering options for the different product materials, two major patterns of cases are formed, and the clustering is generally based on extraction time. In Table 2, a comparison of the ecotoxicity responses (average values for separate clusters) is performed, making it possible to distinguish between different materials, extraction times, extraction media and tests with respect to the registered ecotoxicity. The positive values of ecotoxicity (Y) are an indication of higher toxicity levels and the negative ones (N) of lower toxicity. Ostracodtoxkit F™ registers higher ecotoxicity (especially when "mortality" is taken into consideration) compared to Microtox®. For comparison, a summary of results for the ecotoxicity responses of Xenoscreen tests are also included in the table. Based on the simple guide presented in Table 2 (constructed as a result of over 2000 experiments), one could easily conclude which of the parameters studied are exhibiting specific toxicity levels and risk to infants and children. The overall results of the present study offer, for the first time, specific information about links between ecotoxicity test measurements and experimental conditions imitating real-life situations in the use of products intended for infants and children.

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