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PRELIMINARY STUDY REGARDING SODIUM BENZOATE AND OTHER FOOD DYES SINERGIC ACTION USING BSLA CITOTOXICITY TEST

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Abstract

Legislation enforce allows the use of food additives that have adverse effects on the human body, such as: asthma, contact hives, allergies, digestive disorders, ADHD, cancer. The toxic effect is particularly enhanced when more than one additive is associated in a food. In this context, we propose to study the effect of benzoate ± sorbate on some food colorants (E129 red allura and E133 bluish blue) from 8 fruit juice samples. Sodium benzoate was dosed by the spectrophotometric method using a standard solution Cs = 0.1 mg/mL. Evaluation of the cytotoxicity of these juice additives was followed using the BLSA (Brine shrimp lethality assay) test. For all analyzed samples, the maximum admissible value for benzoate (200mg/L) is observed to be followed. The fastest cytotoxic effects were recorded in the first 24 hours, but at low concentrations of 50 µL/mL and 100 µL/mL. Processes such as cytoplasmic accumulation of inclusion vesicles, disruption of membrane activity, as well as phenomena associated with cell division and differentiation are identified. In conclusion, although all products contain benzoate levels within acceptable limits, there is a risk of accumulation of larger quantities in the body, by consuming products with this preservative. The combination of these preservatives with dangerous dyes increases the toxic potential, which was also confirmed by the BSLA test. Proper product labeling would inform and guide the consumer / patient to balanced consumption with fewer synthetic additives, with the option of choosing an alternative that does not harm the health.

Key words: *cytotoxicity brine shrimp lethality assay (BSLA), sodium benzoate, food colorants*.

INTRODUCTION

Currently in our country, the same additives (natural and synthetic), which are admitted by the Ministry of Public Health and the National Medicines Administration, are used both in the pharmaceutical industry and in food industry in accordance with the EU Regulation 1331/2008 and the recommendations of the Committee joint FAO / WHO expert (EU Regulation, 2008). The safety of food additives in Romania is respected and the limit of benzoic acid in fruit juices of 200 mg/L is established. Great attention is paid to the purity of these additives, knowing they have heavy metals traces (EU Regulation, 2012). With regard to the harmful effect of synthetic food additives, specialists warn that exaggerated consumption of these highly-processed and processed foods can cause a number of harmful systemic reactions: bronchial asthma, contact hives, allergies, digestive disorders, hyperactivity in children, headaches, cancer. If to additive foods are added the additives from drugs, adverse reactions may occur by cumulative effect (Banu et al., 2014).

Benzoic acid is one of the chemical presservatives commonly used as antimicrobial agent in the food and beverage industry refreshing, often used in combination with sorbic acid. There are studies reporting that it is carcinogenic and has side effects such as hives, non-immunological contact hives and asthma (Syed, 2011). Benzene which is carcinogenic can be used at a very low level (ppb level) in products containing both benzoate and ascorbic acid. Exposure to heat and light further stimulates this reaction (Kusi and Acquaah, 2014).

Studies also highlight the toxic effect of many food colorants, such as those commonly found in foods associated with benzoic acid (tartrazine - E 102, Ponceau red 4 - E 124, orange yellow - E 110, carmoisine - E 122,

yellow quinoline - E 104 and red Allura - E 129) (Grumezescu, 2018; Brian, 2014). Benzoic acid in combination with at least one of the 6 colorants has synergistic action for ADHD syndrome, especially among children (Brian, 2014; James, 2014). Many studies have reported this synergy of these additives, which reduces food security for the consumer (Lewis, 2018). In order to improve health safety, the European Union requires that the foods in question be labeled as follows: "the name or E number of the dye (s) may negatively affect the child's care and attention" (Annex V to EU Regulation 1333/2008).

The additives labeling is based on the principle of adequate consumer information, that health is protected and that the product can be used without risk (whether there are allergens, durability, storage conditions or user instructions) (Giurea, 2012). Considering the widespread use in the preparation of soft drinks of these additives that are toxic and potentiates ADHD syndrome, we propose that in this paper we determine the benzoate content in some samples of juices available in the Romanian market and stores and evaluate the cytotoxicity of these additives using the BLSA (Brine shrimp lethality assay) test. The *Artemia salina* bioassay was chosen because it is fast and low cost.

The use of additives in veterinary food and their risk to human population and environment is among the international trends in research (Andreu et al., 2013).

Furthermore, there is a good correlation between in vivo and in vitro tests, and this method is a useful tool for predicting oral acute toxicity. It is also a test that can be used to identify cellular effects of very low concentrations of xenobiotic (Martinov, 2018).

MATERIALS AND METHODS

1. Analyzed samples

Eight samples of fruit juices (Romanian and imported juices) from Romanian market were tested. Juices contain preservatives - benzoate / sorbate and colorants of the hazardous category - red ponceau, brilliant blue and caramel (Table 1). Four samples containing different concentrations (c1-20 μg/mL; c2 = 40 μg/mL; c3 = 100 μg/mL; c4 = 200 μg/mL) of benzoic acid were also analysed test control (control benzoic control test = ABCT) considering that 6 of the analyzed samples include sodium benzoate as well (S3-S8).

Tabel 1. The content of juice sample

Nr. Sample	The content of sample
S1	without preservative, without dye
S2	without preservative, without dye
S3	*B + S; Red allura + Black brilliant
S4	*B +Sr; Red allura+Black brilliant
S5	*B + Blue brilliant
S6	*B
S7	*B + Caramel
S8	*B + Sr
S9	Standard Benzoat

*B=Benzoat; Sr= Sorbat

2. For the spectrophotometric determination of benzoic acid

Following reagents were used: pure benzoic acid Gatt Koller (Germany), Ethyl Ether Sigma Aldrich (Germany), $NaHCO_3 \geq 99.5\%$ (Merck, Darmstadt, Germany); tartaric acid ≥ 99.5%, Sigma Aldrich (Germany), NaOH ≥97.0%, pellets Sigma Aldrich (Germany) and H_2SO_4 98% Sigma Aldrich (Germany).

We used the spectrophotometric method (CAN Standards, 2008; Goma et al., 2013).

Thus, extraction was performed with ethyl ether respecting all the separation steps after eventual neutralization with $NaHCO_3$ and NaOH and dissolution of the filtrate with tartaric acid. The levels of absorbance of ethyl ether were read at 268, 272, 277nm and A_0 calculated according to the formula $A_0 = A_2$ (272nm) - [A_1 (268nm) + A_2 (277nm)] / 2

The sodium benzoate content is calculated using the standard curve, expressed in mg / mL of sodium benzoate.

The calibration curve has good linearity (R2 = 0.9852) and was plotted using the absorbancen values corresponding to the 5 standard solutions prepared, 0.5 concentration; 0.75; 1.00; 1.25; /1.5 mg/mL (Figure 1).

Absorbance readings were performed on the UV 6800PC spectrophotometer; the machine performs triple readings.

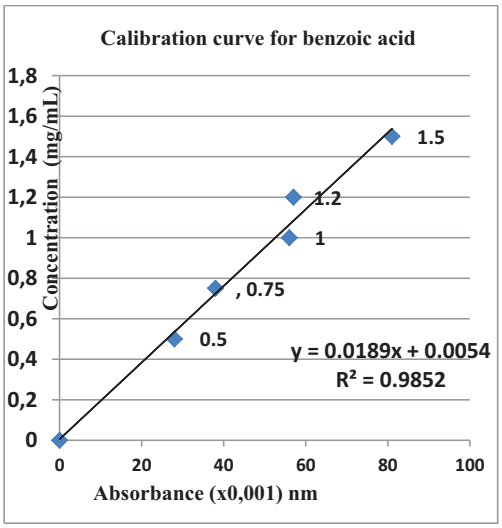

Figure 1. Calibration curve for benzoic acid

Assessment of cytotoxicity

Artemia salina, in naupliar stage I, II was used. These cysts were subjected hatching in artificial seawater of 35-36‰, at 25ºC (thermostat) with continuous aeration and artificial lighting. *Artemia* larvae, hatched about 5 h, were placed in water of 2-3 ppt.

The experimental containers are made of Plexiglas (cell culture plates) with volumes at 1 mL. In each box was placed an equal volume of saline solution with larvae (10-20 specimens/box). Five repetitions were performed for each concentration. Control samples were made by placing the larvae in sea water with salinity like (Andreu et al., 2013).

The quantification of the effects consisted of measuring the survival of the larvae after 24, 48, hour from the start of the experiment. The cytological study was made at Optika B-350 microscope, with Optika Vision Pro photo capture.

RESULTS AND DISCUSSIONS

Following the quantitative analysis of benzoate in the samples taken, all samples were found to be within the maximum admissible values - for juices less than 200mg/L allowed (EU Regulation, 2008). The results obtained are presented in Table 2. For each sample, 3 batches were tested at 1-month interval; from each batch, 3 determinations were made.

The results are expressed as the mean ± standard deviation (SD) of the triplicate determinations.

The ANOVA method and the Student'st test were used to test any statistically significant difference. Correlation values were evaluated using the Pearson correlation. Differences that have $p < 0.05$ are considered significant.

As a result of these determinations, we note that for all samples, the admissible values between 0.062 - 0.200 mg/mL are followed; significant differences are found in samples S3, S5, S6 and S7.

The highest values are in the S5 and S6 samples which have only benzoate, but in S4 and S2 the absence of the preservative is confirmed, as stated on the label.

Although the samples do not contain benzoate in concentrations above the maximum permitted levels, they are not free of risks to the health of consumers (they contain benzoate and dangerous dyes - blue brilliant, red allura, caramel).

Table 2. Values obtained for sodium benzoate from the analyzed samples

Sample	Minimum	Maximum	Mean		Std. Deviation
	Statistic	Statistic	Statistic	Std. Error	Statistic
S 3	0.0570	0.0620	0.059422	0.0005257	0.0015770
S 4	0.1070	0.2050	0.136444	0.0119816	0.0359448
S 5	0.1580	0.1720	0.167111	0.0016368	0.0049103
S 6	0.1380	0.1440	0.140222	0.0007027	0.0021082
S 7	0.0580	0.0640	0.060556	0.0006261	0.0018782
S 8	0.0130	0.1100	0.090222	0.0097721	.0293163

In this respect, there are many studies that report the increasing incidence of ADHD among children in the consumption of products with additives (Lewis, 2018; Rian, 2014; Brian, 2014]. The toxic effect of additives due to synergism from the analyzed samples is also supported by BSLA test results, presented below.

Assessment of cytotoxicity

The effects of the compounds were measured by determining the survival rate of larvae and observing microscopically visible changes. A moderate cytotoxic effect was found in terms of benzoic acid at 24 h and 48 h respectively.

It decreases larval survival at high concentrations of 0.100 mg/ mL and 0.200 mg/mL, respectively.

These observations indicate a nontoxic effect in the first 24h and a very low cytotoxic effect after an extended 48h exposure. For the analyzed samples (P1-P9) the mortality values (%) recorded in the first 24 h suggest significant differences (Figure 2). Mortality over 50% was recorded in P1, P4, P5, P6, P8. These data indicate the cumulative effects induced by the mixture of dye and benzoic acid in P4, P5. In P6, the recorded mortality correlates with the phenomenon recorded in the ABCT test for benzoic acid solutions whose concentrations are above 0.1 mg/mL.

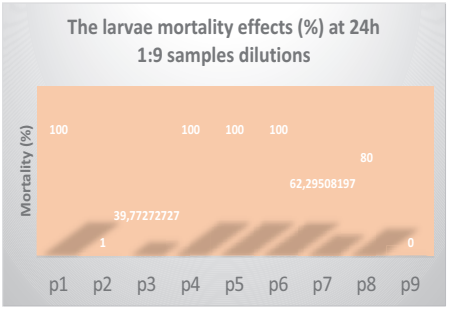

Figure 2. The larvae mortality effects (%) at 24h exposure in solutions samples with 1:9 dilutions

The microscopically analysis

Morphological and cytological changes are evident in all samples. The effects of a larger scale are in the samples containing combinations of benzoic acid solutions and dyes. Effects identified based on microscopic observations were classified into two categories:

1. Visible effects at the cellular population (limb germs, subcuticular epithelial layer) (Figures 3, 4). Visible changes are noted for sample S3 and S4. These results are explicable, since S3 contains benzoate and sorbate; even if the benzoate is in a lower concentration than the other samples. Sample S4 has high benzoate values but also two red dyes (red allura and Blue brilliant)

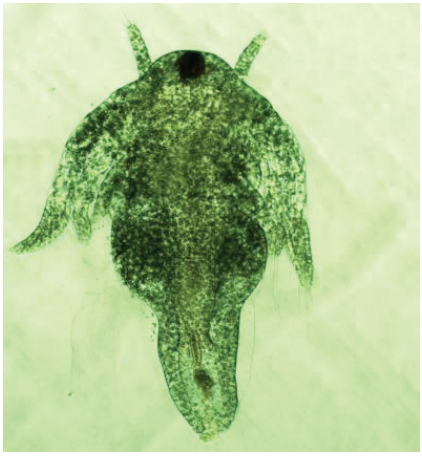

Figure 3. Abdominal and digestive tract morphological changes for P4 sample

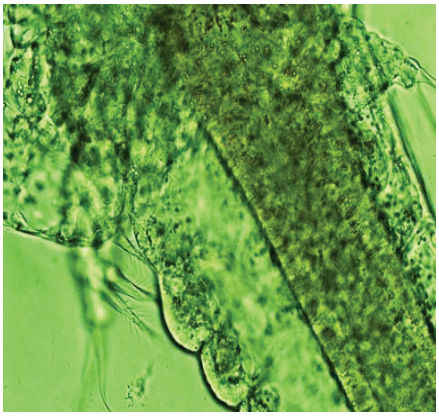

Figure 4. The limb germs inhibitions for larvae from S3 sample

2. Intracytoplasmatic, highlighting numerous cytoplasmic vacuoles, identified for S4 - Figure 5. Some larvae are deformed and the explanation is probably related to osmotic perturbations (Figure 3), but the larvae continued to survive for several hours. The most important cytological phenomena noted were those related to inhibition of organogenesis (Figure 4). These observations also explain the increased mortality of larvae exposed to the tested solutions. Theoretically, in the first 24-48 h, the larvae pass through 2 or 3 successive moults, during which time divisions and respective cell differentiation processes occur that ensure success during growth.

A similar phenomenon has also been described for invertebrates, in literature. Thus, Martinov's

study, 2018, indicates inhibition of growth and reduction in the number of moults in *Tenebrio molitor* larvae.

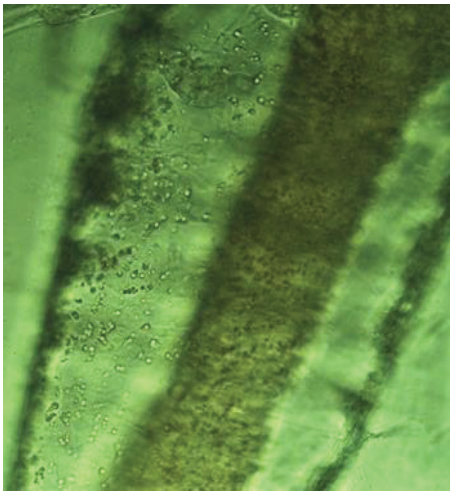

Figure 5. The cytoplasmatic vacuolization's for P4 sample

Phenomena such as growth inhibition and cytokine synthesis, glycolysis alterations are noted in other testing systems such as human lymphocytes (Freeman, 2005) or *Saccharomyces cerevisi*ae (Bruno, 2006) at exposure to benzoic acid solutions.

Cytological studios highlight phenomena that can be used later to understand the mechanisms of action of widely used additives in the food or human food sector.

Extensive morphological changes such as inhibition of cell growth and disruption of membrane integrity are those that explain the decrease in Artemia larva survival after 24 hours of exposure to dyes or admixtures.

As a result, our study suggests that the way conservatives and dyes are associated, would require more attention when establishing the need for additives in veterinary or human food.

CONCLUSIONS

Following BSLA cytotoxicity tests for fruit juices containing benzoate / sorbate and dangerous dyes responsible for a range of phenomena in humans and animals, the results show that:

• intensively processed products still jeopardize food safety and, implicitly, the health of the body;

• better nutrition education with the involvement of competent institutions and parents is needed if we refer to children;

• cytological changes suggest that additional studies are needed on how benzoate / sorbate combinations and dye combinations work at morphological and cytological level;

• identifying the phenotypes of blocking growth or cytoplasmic functioning or osmotic perturbations could also explain a series of phenomena observed in humans;

• products containing a chemical substance used as additives continue to jeopardize the safety of veterinary and human food;

• these chemical combinations may represent a major risk to human health and the environment.

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