Analysis of the influence of polystyrene microplastics and their derivatives on the DNA of human colon epithelial cells HT29 with the comet assay

Paweł Filipkowski (pawel.filipkowski@pg.edu.pl)
Gdańsk University of Technology
Ćwiklińska Aleksandra
Gdańsk University of Technology
Zuzanna Koziara
Gdańsk University of Technology
Karol Parchem
Gdańsk University of Technology
Łukasz Zedler
Gdańsk University of Technology
Jacek Ryl
Gdańsk University of Technology

Article

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Abstract

We have been using plastic for almost a century and nowadays a lot of them circulates as pollutants in the environment and still defragmenting to micro and nanoscale. The exposition through the food chain and its precise impact on human health is still not clear. In our study, we tested real food packaging after contact with food products and real thermoinsulation and environmental samples of polystyrene in different model liquids, We used a comet assay, mass spectrometry (MS) of fluids which were in contact with packaging, free styrene measurement by Wijs method according to ISO standard and FTIR of wrap, to get an overview of their possible harmful effects.

In the presented study, only low genotoxic effect (5-6% DNA damage) was observed.. In addition, no adverse effects were detected in the mass spectrometry. For analysed coloured polystyrene food packaging materials, we noticed a lower level of free styrene monomers in dairy packages that originally contained products with fruits. Exposure of cells to long-term seasoned polystyrene caused less DNA fragmentation than in the case of samples in saline.. Unfortunately, to this day no one knows how much free styrene and derivatives we eat with our daily food products and from food packaging.

Based on our results and knowledge, more attention is needed with regard to polystyrene contamination. In particular, a wider analysis of the impact of interactions with the microbiota and the food matrix, which is in direct contact with the polystyrene packaging, should be carried out.

The results of our research show that polystyrene and its derivatives from food packaging can potentially have a negative effect on the DNA of human colon epithelial cells. We recommend seasoning of freshly formed polystyrene products and increasing personal protection in relation to workers of the polystyrene foaming factories This applies primarily to dairy products, readily consumed by children, which are rich in both organic acids and salts, and the presence of these ingredients enhances the genotoxic effects. According to our best knowledge, this is the first study using actual polystyrene food packaging available on the market.

Introduction

Plastic products are widely used in many areas of life, including agriculture and industry. Due to the fact that they are characterized by lightness and low production costs, they have been replaced glass, wood, etc. However, plastics can fragment, which leads to the formation of small plastic particles – materials with a diameter of less than 5 mm and are known as microplastics (MPs). We can distinguish between primary MPs, usually found in textiles, personal care products and medicines) and secondary MPs created as a result of physicochemical and biological effects of degradation). MPs also come from other sources: e.g. from abrasion of car tires or various types of coatings.[1]

Plastics are synthetic organic polymers formed by the polymerisation of monomers extracted from hydrocarbons. Plastics and MPs enter the soils and oceans and then accumulate in the marine environment and are considered to be one of the most common and persistent pollutants.[2]

Polystyrene (PS) was first used industrially in Germany in the mid-1930s. Since then, it has been widely used in the production of packaging, for example for storage and food (in developed countries 50-60% of PS production volume is used for food packaging, in the United States 14% and in Japan 10%). It has desired properties such as high processability, shape repeatability and excellent foaming ability. PS products contain styrene oligomers formed as byproducts during the polymerization process. The literature confirms that styrene dimers and trimers can migrate from polystyrene products.[3] PS containers are formed by gas injection into PS masterbatch melts, resulting in a relatively loose structure and a rough surface.[4]

Well known definition *Food and Agriculture Organisation of the United Nations (FAO)* says*: 'food security is a situation that exists when all people, have physical, social and economic access to sufficient, safe and nutritious food that meets their dietary needs and food preferences for an active and healthy life'* (FAO 2002). The *four main pillars of food security can be mentioned: availability, access, utilisation and stability of food (FAO 2009).* The presence of MPs in marine organisms threatens two above pillars: food availability and utilisation. After ingestion of MPs, marine organisms are unable to break down the polymers, meaning that MPs can be retained and accumulate in their body. This blocks elements of the digestive system, causing food digestion to be impaired. MPs move through the food web and bioaccumulate and biomagnitude in higher organisms.[2]

In theory, human skin prevents MPs and other pollutants from penetrating it. MPs and other contaminants can penetrate sweat glands, hair follicles and wounds causing skin breaks. However, it is recognised that plastics from seafood and the environment itself may pose a greater risk due to interactions with other contaminants, leaching of plastic additives or weathering. So, even if we stop using plastics right now (which is impossible in real life), we would be exposed to them for years yet.[2]

Plastic debris is a serious threat to our planet. According to estimates, about 10% of plastic ends up in the seas and oceans as a result of its poor management, and recycling rates are still too low. It was estimated that more than 5 trillion plastic particles are floating on the surface of the oceans. Plastics account for approximately 92.4% of all marine pollution. Plastics are slowly degraded by microorganisms, sunlight or mechanical abrasion into secondary MPs. Most of the literature data presented up to date suggest that MPs take up to several thousand years to degrade further.[5]

Our present level of knowledge about 'nonbiodegradable, durable PS' in face of facts about how MPs are reated and changing.[6] There are still new publications about the tissue and organs where MPs were ound and especially the toxicity of MPs in a mammal where presented here research fulfil lacks.[7]

)n the other side, there is no reliable and sufficient information about the accumulation of PS. However, here are data on the degradation of PS at the level of 1% within 90 days in soils containing nicroorganisms and Eukaryota.[8]

As very latest research shows, plastic waste freely circulating in nature are fragmented to MP and nanoplastics size of particles and could be accumulating in the environment and in a higher organism. There is a lack of control, precise data and legal regulations regarding MPs circulating in the environment.[9]

Moreover, it seems that all of that stable and non-degradable (as we still thinking) plastics could be found in every tissue and after all the natural barriers like it was shown in mouse brain.[10,11] Kik et all in 2020 proved polystyrene nanoparticles accumulate along the food chain. They mentioned to about law amount of studies focused on human organism too. And what is more disturbing, everything shows that MNP and their derivatives have an impact on the cells of living organisms. Researchers report some toxic effects, problems with the development of new neurons or on the contrary acceleration of the apoptosis of damaged neurons.[11,12]

Data showing a negative effect on marine organisms like fishes are widely spread and of concern. After only twenty days of exposure to MP of PS, there were noticed transcriptomic influence, some immune response and some down-regulations of a few genes and changes in the behaviour of zebrafish.[13]

What seems terrifying in 2022 Leslie et al. proved that some MNPs were detected not only after the brain barrier, but freely moving in human blood too.[14]

What Wang and co-researchers prooved in 2021 and 2022 on nematode model that intakes PS nanoparticles (especially in a case of long-term exposure and with oxidative stress) influence negatively on expressions of some methyltransferase genes. And even known repair system doesn't work properly.[15–18]

Another known facts to consider when we are talking about MPs is that they are not lonely particles in the environmental and food matrix. All MPs are already well known and described as very good absorbers. They can interact with: heavy metals, PAHs, PCBs, OCPs, PBDEs, pharmaceuticals, toxins, endocrine disruptors, mutagens etc. Considering the interaction with other particles in the environment, MPs are a perfect vector of many potentially toxic xenobiotics.[2,19] Factors such as UV radiation, the presence of salt ions and wave movement in the marine environment, as well as contact and exposure time, contribute to the increasing fragmentation of plastics and an increased risk to human health. [5] Moreover, so far no one has been able to estimate how these compounds adsorbed by MPs are released in our digestive tract and which changes in our body they undergo. It has been proven that other plastic derivatives, such as bisphenols, can only become dangerous in our digestive tract. Katsumiti et al. used a model involving the lysosome, to show how important is the size of the MPs itself and how far-reaching the consequences of their accumulation may be, proving that MPs can act as a carrier of benzo-a-pyrene to mussel hemocytes.[20]

Traditional synthetic polymers are usually reported as highly resistant to microbial degradation and this is the main problem when such polymers go into the environment. PS is one of the most widespread

polymers, but knowledge about its biological degradability and influence on human living cells, especially on their DNA, is scarce.

In 2020 and 2021 Domenech and coresearchers noticed that even the type of human cell lines and coactivators (like the coexistence of other cell lines or ROS generators) could have an impact on the response of cells to the presence of MNPs. And as it was suggested another intestinal cells line, like e.g. HT29, should be used and checked.[10,21]

In most of the published data authors take care about the not-so-high level of PS or its oligomers. Usually, in that point of view, the level of genotoxicity is shown as marginal. Clift's experiments with nanoparticles in 2013 and Nakai's experiments with food packaging in 2014 on PS and derivatives led to the following conclusions: 'no significant influence', with or without metabolic activation (in the Ames test). The *in vitro* chromosomal aberration test with Chinese hamster lung cells (CHL/IU) wasn't 'worrisome' too. Both mentioned above authors' results suggest that the risk of the genotoxicity of styrene oligo and lower mers that could migrate from food packaging or thermal insulation is 'very low'.[3,22] On the other side, there are reliable data proving that MNPs could go freely through any biological membrane.[10]

More information is needed to determine the potential risk of MPs to human health. Currently, few studies using human cells can be found to assess the environmental impact of MPs. A wide variety of human cell lines can be used to determine the adverse effects of MPs, and it seems reasonable to use the cells most exposed to this factor. Given that an important route of exposure is the gastrointestinal tract, the use of intestinal cells is appropriate approach.[23]

In the presented study, we used SEM microscopy, AT-FTIR, the Wijs method and mass spectrometry. We also used the comet assay to determine the genotoxicity of PS from real food packaging and thermoinsulations materials in the model liquids on the HT29 cells. In contraty to the presented up-to-date publications for other human cell lines, we noticed around 5-6% of DNA degradation, and in our opinion researchers should focus more on these minor changes.

Results

Scanning electron microscopy (SEM)

Before treating MP of PS in model solution and after disintegration in steel ball mill, shape and size of prepared samples were assessed with SEM. Results are shown below.

The edges of the fragmented real food container of homogenized cheese/vanilla yoghurt look more plastic (not so sharp) than samples where pure PS without any additions and prior processing such as foaming are. The SEM study of the topography of the samples confirms the presence of a whole range of 'micro' particles. The size distribution of the milled microparticles is similar regardless of their origin.

HP-LC-Q-Orbitrap HRMS analysis

MS analysis of water incubated at 36.6°C for 4 weeks in lunch or soup containers made of expanded PS did not show the presence of PS di- or trimers. We generally do not see any significant release of monomers or derivatives to the water in the package in performed MS analysis in reasonable concentration.

Fourier transform infrared spectroscopy (FTIR)

Spectral analysis of tested real food container and other materials were done. Exampled spectra are presented in the the figure 2.

Fig. 2 shows the FTIR spectra of three selected polystyrene-based products: real nono-coloured food packaging (yoghurt), seasoned thermal insulation (3 years), and freshly foamed polystyrene. Typical bands for PS can be observed on all spectra. Two small peaks in the range of 3061 cm^{-1} and 3026 cm^{-1} correspond to the aromatic C-H stretching vibration absorption, while peaks at 1600, 1493, and 1452 cm^{-1} are due to aromatic C=C stretching vibration absorption showing the presence of benzene rings in the structure. Moreover, the presence of bands at 750 cm^{-1} and 694 cm^{-1} are due to C-H out-of-plane bending vibration absorption, which shows that the benzene ring has only one substituent.[24,25] The visible bands at
2848 cm^{-1} and 2915 cm^{-1} correspond to the existence of methylenes in the PS structure. The only significant difference between analyzed samples are two peaks at 2356 cm^{-1} and 2337 cm^{-1} corresponds to the presence of CO_2 encapsulated in expanded PS. According to the presented spectra, there are no significant dissimilarities between each other, while the difference in the intensity of peaks results from the sample preparation and the thickness of tested specimens.

Determining of the free styrene monomer by the Wijs method

The amount of free styrene monomers from real food packaging and thermal insulation materials was determined using the Wijs method in accordance with the ISO standard. We observed a much lower level of free styrene monomers in seasoned thermal insulation materials (fig. 3a). For tested and presented coloured PS food packaging materials, we noticed a lower level of free styrene monomers in dairy packages (from the same manufacturer) that originally contained products with more fruits (fig. 3b).

DNA damage assessed by the comet assay

We checked the influence of MPs released from foaming substrate, freshly expanded and 3 years seasoned PS on the DNA integrity of HT29 cells. Images of few representative comets are shown in the fig. 4 and the results of the comet assay are presented in the fig. 5a.

The results are in line with those obtained in the Wijs method. Exposing cells to ‘older’ PS (3 years seasoned) caused less damage, and the presence of salt promoted DNA fragmentation. A similar influence is noticed in the use of lipids/olive oil as a PS MPs preparing medium, which is presented in the fig 5b.

Similar level of average DNA damage was observed in the case of, commercially available at the market, coloured food packaging from dairy products (after removing food product). Interestingly, the effect of final concentration in well with cell, 1.5% ethanol and 0.3% acetic acid, was more pronounced than that of lactic acid, which is naturally present in dairy products (fig. 6).

Discussion

Plastics, even with some law changes in the EU during the last few years, are still widely used. Aspects such as the circulation of plastics in nature, their durability or ways of influencing our body should draw more attention from researchers to the associated risk. Results obtained by the researchers suggest that MPs accumulate in higher organisms, and moreover, they are becoming more and more fragmented, even to the nanoscale. Furthermore, a lot of authors proved that MPs could be a very good vector of the dangerous compound into our body and cells and freely goes against all the human natural barriers. All obtained results also suggest that MPs are not as indifferent as we thought so far.[2,19,26] Polymers are one of the most important sources of environmental pollution. Plastics degrade to the size of MPs, while creating invisible environmental pollutants. Humans can be exposed to MPs through the food chain or through other routes, such as air or tap/bottled water. They pose a real risk to human health, regardless of the route of exposure.[23]

The RP-LC-HRMS analysis inditated that the incubation of destiled water in the lunch box or container purposed to use for soup produced from expanded PS at 36.6° for a period of time up to four weeks did no significant increase in the amount of detected ions compared to the control destiled water not incubated with this material. The analysis of distilled water which was in contact with PS packages did not provide information about the presence of any larger particles migrating from the package - neither dimers and/or trimers. Moreover, no lower
*-mer degradation products, e.g., formed as a result of oxidation, were found in the analyzed samples. In our research, we used a wider range of model solutions beyond those recommended in the Directive of the Council of the European Communities of 19 December 1985 (85/572/EEC), such as: lactic acid solution, and higher concentrations of ethanol (40%). We examined real food packaging (after removing food product) and real thermal insulation materials at a different stages of processing (substrate, freshly expanded and 3 years seasoned). Courtesy of prof. M. Niedostatkiewicz (Gdańsk University of Technology, Faculty of Civil and Environmental Engineering, Department of Engineering Structures) we also obtained the buoys of fishermen from the vicinity of Gdańsk Bay - part of the Baltic Sea (fig. 7).

n FTIR analysis we observed more CO bonds at around 2300 cm^{-1} in fresh expanded PS, and lowering evel of detected signal at 3000cm^{-1}. The only significant difference between analyzed samples are two eaks at 2356 cm^{-1} and 2337 cm^{-1} and it probably corresponds to the presence of CO_2 encapsulated n freshly expanded PS (fig. 2). More compound could be find in colatile fraction as Marć described reviously.[27] The release of less free substances and the stability of the polymer also influenced the

results obtained in the comet assay and the technique of free styrene determination with Wijs reagent (fig. 3a and fig. 5a)

One of the exceptions in this pattern is polystyrene sea buoys. Results obtained in our experiments with food packages and other materials made from a EPS (like mentioned above sea equipment or thermal insulation materials) definitely prove that the presence of NaCl (but also lipids) increases the amount of free styrene recorded and the level of DNA damage in cells (fig. 4 and fig. 5b)

Interestingly, even for cells treated with MP obtained from 3 years seasoned expanded PS from thermal insulation materials, the level of DNA damage was greater than for control cells. (fig. 5)

Comparing the selected plastic materials (fig. 5 and fig. 6), it can be seen that the average level of DNA damage in HT29 cells is low. The maximum level of fragmentation is 5-6% of the DNA in comet tails. Comparing it to control or contrary to a typical genotoxic compound (like H_2O_2 – used in positive control) it doesn't look alarming. Other authors, examining MP using the Ames test or using Chinese hamster tissue, in their work called it strictly 'worrisome'[3,6,8,22].

In our opinion, it is a very dangerous point of view. Firstly, we tested MPs in typical concentrations and in fact only with a quite short time of contact with cells (24h), but nowadays we should consider MPs as a component which is constantly presented in our digestive tract. What will happen if the exposure/contact time is practically endless? In 2021, Domenech et al.perform long term (8 weeks) experiment. They used synthetic PS nanoplastic and Caco-2 cells in clear laboratory conditions and observed a similar level of damage.

It is worth underlining that MPs are not alone in food or the environment[28]. Furthermore, food containers are usually mixed with additives like colourants. Secondly, the model liquids that we tested are commonly used in the food industry. We have to remember that in our GUT MPs are exposed to much more extreme conditions and as already mentioned. In our results, we can confirm the presence of salt promotes higher DNA damage. And thirdly degradation has an impact on properties of materials, but we do not know in which way.

A study carried out by Guimarães et al. showed that PS nanoplastics even at low concentrations, can lead to DNA damage in erythrocytes. This results in clastrogenic and molecular damage. The most common DNA damages are those originating from single and double DNA strands breaks, DNA adducts or DNA-)NA and DNA-protein cross-linking, which most likely result from interactions between the contaminant nd the DNA. These mechanisms are consistent with the type of damage often reported in model ystems that are subjected to the comet assay.[29]

Some DNA damage does not look like a typical comet. For some nuclei, the flow of DNA can be)bserved as if the permeability of the membranes was disturbed - especially in salt environment (fig. 4 eft lower corner)

Let's go further. What happens if organisms are constantly and for a long time exposed to some factor? Usually, they adapt. After many years of thinking about plastics as a very durable and problematic element in environment, we could notice a rising amount of information about enzymes, microorganisms or even a higher organism like worms (of course with their microbiota) which could utilise plastics.[13,30]

So, if we know that microbiota could interact with MPS particles, the presence of dyes and fire retardant additives weakens the structure of PS, and MPs are a perfect vector of xenobiotics into our body, maybe we should start to focus on MPs more intensively? We shouldn't underestimate this 'small' 5-6% of DNA damage which we observed in the comet assay. There are known data about the real negative influence of MPs and derivatives on all aspects of the life of e.g. on zebrafish, mice etc.[11,31]

Additional things to consider should be the impact of the consistency of the food matrix (like for example antioxidants and activity of microbiota) on the inner surface of food packaging. When we compare results obtained in determining free styrene and DNA damage we could find some slight differences for a different types of yoghurt – with or without fruits and the level of damage and determined free styrene in packaging from real-life available to buy food dairy products. No one knows how much free styrene and derivatives we eat with elements of our daily food products (fig. 3b and fig. 6)

Our 5-6% of DNA damage is only one brick in the wall. There are fresh data about promoting toxicity of MNPs in the presence of oxidative stress factors as an toxicity inductor.[16,20] According to FAO regulations food should be first of all safe. The migration of styrene monomers in food and food contact materials (FCMs) is dangerous, although the PS products themselves are considered harmless. In a study by Hwang et al. it was confirmed that PS particles were not toxic to human cells at an experimental dose of about 500 μg/mL. PS particles with diameters of 10-100 μm were not significantly cytotoxic. In contrast, smaller PS particles (460 nm and 1 μm diameters) infected red blood cells RBCs. The adhesion of small PS particles to RBC) was enhanced by weak e.g. van der Waals forces, leading to hemolysis.[32]

PS do not induce adaptive immunity and do not cause an increase in histamine secretion. Therefore, PS did not trigger an allergic reaction or histamine-mediated inflammation. Uptake of PS particles occurred mainly by endocytosis and phagocytosis by phagocytic cells. This induced the release of pro-inflammatory cytokines that caused local inflammation. It seems smaller PS particles are not toxic to various human cells, but direct contact with RBC could cause hemolysis, and PS particles in higher concentrations could induce early-stage inflammation.[32]

When we put together information about constant presence and fragmentation, easy penetration via membranes, accumulations along the food chain, surroundings by protein or other xenobiotics corona, influence of seasoning of PS materials, important role of ROS an understanding human exposure in humans we must ask ourselves the fundamental question: what is the risk for public health …and what can we do with that?

Conclusion

In our study, we tested real food packaging after contact with food products and real thermo insulation and environmental samples of PS in different model liquids and conditions with a few analytical and biological methods. Based on our results and collected information we conclude that more attention is needed with regard to PS contamination, and especially a wider analysis of the impact of interaction with the microbiota. Seasoning of freshly formed PS products plus increased personal protection in relation to people working in PS foaming factories should be considered. A broader analysis of the nutritional matrix that comes into contact with PS packaging should also be carried out - we don't really know how much PS and its derivatives we eat and what real impact it has on our health. And it definitely could have a negative impact on the DNA of our colon epithelial cells. This applies primarily to dairy products eagerly consumed by children - and these products are rich in both acids and salts - and as our results show, they enhance the genotoxic effect.

Methods

Chemicals and reagents

The following compounds were used: analytical grade ethanol and methanol (POCH, Poland), DMSO, hydrochloric acid (HCl), low melting point agarose (LMP agarose), sodium chloride (NaCl), sodium hydroxide (NaOH), ethylenediaminetetraacetic acid (EDTA), 2-amino-2-(hydroxymethyl)-1,3-propanediol (Trizma-Base), SYBR®Green I nucleic acid gel stain, Triton X-100 (Sigma-Aldrich, USA normal melting point agarose (NMP agarose) (Bioline, UK). All reagents utilized in the cell culture (PBS, McCoy's 5A medium, trypsin, foetal bovine serum, antibiotics: streptomycin, penicillin) were purchased from Sigma-Aldrich (USA). PBS solution was prepared by dissolving one tablet in 200 mL purified water. Reagents for electrophoresis were from Sigma-Aldrich (Taufirchen, Germany). All compounds for the determination of free styrene in the Wijs method were from WarChem (Poland). QPLUS185 system from Millipore (USA) was used for water purification. Not listed chemicals and biochemicals were purchased from Sigma-Aldrich and were of the appropriate grade. Main of tchem were purchased and used as it was described before.[33]

Samples preparation

The solutions used in the study comply with the Directive of the Council of the European Communities of 19 December 1985, which establishes a list of model liquids for testing the migration of constituents of plastic materials and other articles intended to come into contact with food (85/572/EEC). The simulants used in the study were: distilled water, a solution of 3% acetic acid, an aqueous solution of 15% (v/v) ethanol and rectified olive oil. Additionally, we used 2% of lactic acid – the main acid in dairy products. (EU directive 85/572/EEC). Testes materials and used model liquids are listed in tab. 1. All plastics were disintegrated using a mini ball mill with a steel chamber (27 mm diameter) and one stainless steel ball (10 mm diameter) (Vibrator DDR-GM9458) Germany). The size of the particles was analysed with SEM microscopy (fig. 1). Then MPs were kept in model solutions for 24h, and after that, we allow affect on the DNA of nuclei growing for 24 hours of HT29 cells.

Tabl. 1. Analysed PS materials and model liquid solutions.

	Take-away packaging			Dairy products packages	Styrofoam (EPS)			EPS buoys
	lunch package	soup package	mug		EPS substrate	fresh EPS	seasoned EPS	
H_2O dist.	+	+	+		+	+	+	
2 % Lactic acid				+				
3 % Acetic acid	+	+	+	+	+	+	+	
15 % EtOH			+	+				
0.9% NaCl					+	+	+	+
Olive oil	+							

Genotoxic effects determined by comet assay

All performed experiments were done with guidelines from MIRCA.[34] HT29 cells were seeded in 24-well plates (200,000 cells/well in 1.8 mL of medium) and was incubated for 24 h at 37°C. Next, the cells were treated with 200 µL of prepared microplastic solutions in the specified medium. The final concentrations of model liquids in the wells were 0.2 %, 0.3%, 1.5% and 0.09% (v/v)for lactic acid, acetic acid, ethanol and NaCl solution, respectively. A control with the appropriate medium was also prepared. 3 technical replicates were performed. The plates were incubated for 24 hours. One hour before the end of incubation, 3 wells were treated with 1,5mM H_2O_2 – freshly prepared from 30%/~10M stock (positive control).

A 0.5% LMP (low melting point) agarose solution was prepared (0.05 g agarose was added to 10 mL PBS and liquefied), dispensed into tubes (150 µL each) and placed in a thermoblock heated to 40°C for 30 min.

After the cells incubation, fluid was removed from all wells with an aspirator and cells were washed with warm PBS (37°C). A fresh portion of PBS was added to the wells (2 mL per well)„ cells were scraped from the wells and 1 mL of the suspension was transferred to tubes placed on ice. The cell suspension was centrifuged (1000 rpm, 5 min, 4°C). The supernatant was removed, so that approximately 50 µL of liquid remained at the bottom. 30 µL of the suspension was transferred to the tubes containing LMP agarose and briefly vortexed. Then, 30 µL of the suspension was applied as two spots on a microscope slide pre-

coated with 1% normal melting point agarose (NMP agarose) covered with coverslips and transferred on ice immediately. After 10 minutes, the coverslips were gently removed from the gels and the slides were transferred to lysis buffer (2.5 M NaCl, 0.1 M EDTA, 0.01 M Tris, 1% Triton X100, pH 10) for 24 hours at 4°C. After incubation, the slides were transferred to an electrophoresis apparatus filled with electrophoresis buffer (0.01M EDTA, 3M NaOH, pH>13, 4°C), which was previously placed on ice.

For chromatin unwinding, slides were incubated in the dark for 20 minutes and after that electrophoresis was conducted (voltage 26 V, constant current 300 mA) for 30 minutes, maintained at 4°C. After the electrophoresis, the slides were transferred to cold (4°C) PBS and incubated for 5 min at 4°C. Subsequently, the buffer was replaced with a fresh one and the incubation was repeated under the same conditions. After removing the buffer, incubations were performed with water and then with 70% ethanol (under the same conditions). Slides were taken out and left out of light to dry. Then, slides were stained on the shaker for 30 min with cold (4°C) Sybr Green dye solution in TE buffer (1 mM EDTA, 0.1 M Trizma-base, pH 7.5) and gently rinsed for 10 min with distilled water. The wet gels were covered with coverslips, and subjected to further analysis. DNA comets were examined under a fluorescent microscope (Carl Zeiss AxioImager Z2) connected to a scanning system Metafer 4 (MetaSystem, Zeiss) and digital imaging camera (CoolCube1). Then, 200 consecutive comets were counted for each repetition and slide. % DNA in Tail parameter was used to express DNA damage. Comet counting and analysis were carried out Metafer and Comet Score Pro software (TriTek Corp. VA, USA)

Cell Culture

HT29 cell line (human colon adenocarcinoma) from the ATCC was used as a model of the human intestine. Cells were maintained in McCoy's medium with the adition of antibiotics (100 U/mL streptomycin and 100 g/L penicillin) and foetal bovine serum (100 mL/L). The HT29 cell line was keept at 37 °C under a controled 5% CO2 atmosphere in the incubator (Heal Force) .[33]

Determining of the free styrene monomer by the Wijs method

For the determination of free styrene, a 3 g part of EPS or the container made of food-grade PS was cut off and fragmented into small pieces and further dissolved in chloroform into a 100 mL measuring flask in accordance with the guidelines contained in the standard ISO 4901:1985 and ISO4901:2011 (based generally on ISO/R173:1961) number. Then 25 mL of the prepared solution was measured into a 200 mL conical flask, 5 mL of Wijs reagent (the solution of dissolved iodine trichloride and iodine in a mixture of concentrated acetic acid and chloroform – WarChem, Pl) was added and left for 15 minutes in a dark place. After that added KI solution (1 %, v/v, 5 mL), distilled water (20 mL) and starch indicator (1 %,v/v, mL – WarChem, PL). The released iodine was titrated with sodium thiosulphate solution until the olution was completely discoloured. The determination was performed at least 3 times. Also, a blank was performed following the same procedure as above, but using 25 mL of chloroform in the place of a ample.

Iodine trichloride and iodine react with each other to form iodine chloride. The styrene in the sample reacts with iodine monochloride. The portion of iodine chloride remaining after reaction with styrene then reacts with potassium iodide to form iodine. In the final step, the released iodine is titrated with a sodium thiosulphate solution (against starch as an indicator.

Scanning electron microscopy (SEM)

A FEI Quanta 250 FEG (Thermo-Fisher Sci.) equipped with a Schottky field emission gun was used to perform scanning electron microscopy (SEM) micrographs. The microscope worked with secondary electrons, under a 20 kV accelerating voltage and in high vacuum mode.

Fourier transform infrared spectroscopy (FTIR)

To gather more data about the state of polymer, Fourier transform infrared spectroscopy (FTIR) analysis was performed. The FTIR of the analysed polystyrene materials was carried out with a Nicolet Spectrometer IR200 from Thermo Scientific (Waltham, MA, USA). The device had an attenuated total reflection (ATR) equipment with a diamond crystal. Measurements were performed with 1 cm−1 resolution in the range from 4000 to 400 cm−1 using 64 scans.

RP-LC-Q-Orbitrap HRMS analysis

The samples were analysed using a Dionex Ultimate 3000 UHPLC system (Thermo ScientificTM, Dionex, San Jose, CA, USA) equipped with a Kinetex XB-C18 column (50 × 2.1 mm) filled with 1.7 µm particles (Phenomenex, Torrance, CA, USA). The mobile phases were composed of acetonitrile/water (50:50 v/v) with 10 mM ammonium formate and 0.1 % formic acid (phase A), and isopropanol/acetonitrile/water (85:10:5 v/v/v) with 10 mM ammonium formate and 0.1 % formic acid (phase B). The gradient program was set as follows: linear increase from 32 % to 70 % B at 11 min followed by a linear increase to 97 % B at 18 min, which was isocratically held for 12 min (30 min). Finally, the initial mobile phase composition was set and the column was equilibrated for 6 min. The mobile phase flow rate was 260 µL/min and the column temperature was 50 °C. The injection volume was 5 µL.

The chromatographic system was coupled to the Q ExactiveTM Focus Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a heated electrospray ionisation source (HESI II). The ionisation parameters in positive ion mode were as follows: spray voltage +3.5 kV, capillary temperature 230 °C, heater temperature 300°C, sheath gas (nitrogen) flow rate 45 arbitrary units (AU), auxiliary gas flow rate 15 AU, and S-lens RF level 35. The full-scan analysis parameters were as follows: resolution 70,000, AGC target 1e6, max injection time 100 ms, and scan range 200 – 2000 m/z. The data-dependent MS2 parameters were as follows: resolution 17,500, isolation window 3.0 m/z, and normalized collision energy 30. The mass spectrometer was tuned and calibrated using a calibration solution (Thermo Scientific) containing n-butylamine, caffeine, Met-Arg-Phe-Ala (MRFA), and Ultramark 1621.

Statistical analysis

All values represent means±SD of at least three independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA) with Prism 4 (Version 4.0c, GraphPad Sofware Inc.).

Abbreviations

CA – comet assay

EPS – expanded (foamed) polystyrene

FTIR – Fourier transform infrared spectroscopy

LMP – low melting point agarose

MP – microplastic

MNP – micro-nano-plastic

NMP – normal melting point agarose

OCP – organochlorinated pesticides

PAH – polycyclic aromatic hydrocarbons

PBS – phosphate buffer

PBDE – polybrominated diphenyl ethers

PCB – polychlorinated biphenyls

PS – polystyrene

RBC – red blood cells

ROS – reactive oxygen species

SEM – scanning electron microscopy

Declarations

Acknowledgements

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Author contributions

P.F., A.Ć. planned the experiment. A.Ć. carried out the main experimental part. P.F. supervised the experiments. P.F., A.Ć. analysed the data, carried out the statistics and prepared the tables/figures. A.Ć. wrote part of the draft manuscript. P.F. wrote the final version of the manuscript. Z.K. helped, teach, and take care of, especially at the intial stage of comet assay experiments and revised manuscript. K.P. performed a mass spectrometry experiment and analysed data. Ł.Z. performed FTIR experiments and prepared fig. 2. J.R. carried out the SEM experiment and prepared fig. 1. All authors have read and agreed to the published version of the manuscript.

Data availability statement (mandatory)

Data are available on request from the P.F.

Competing interests

The authors declare no competing interests.

Additional information and any of supplementary information is available for this paper at

correspondence and requests for materials should be addressed to P.F. (pawel.filipkowski@pg.edu.pl)

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Figures

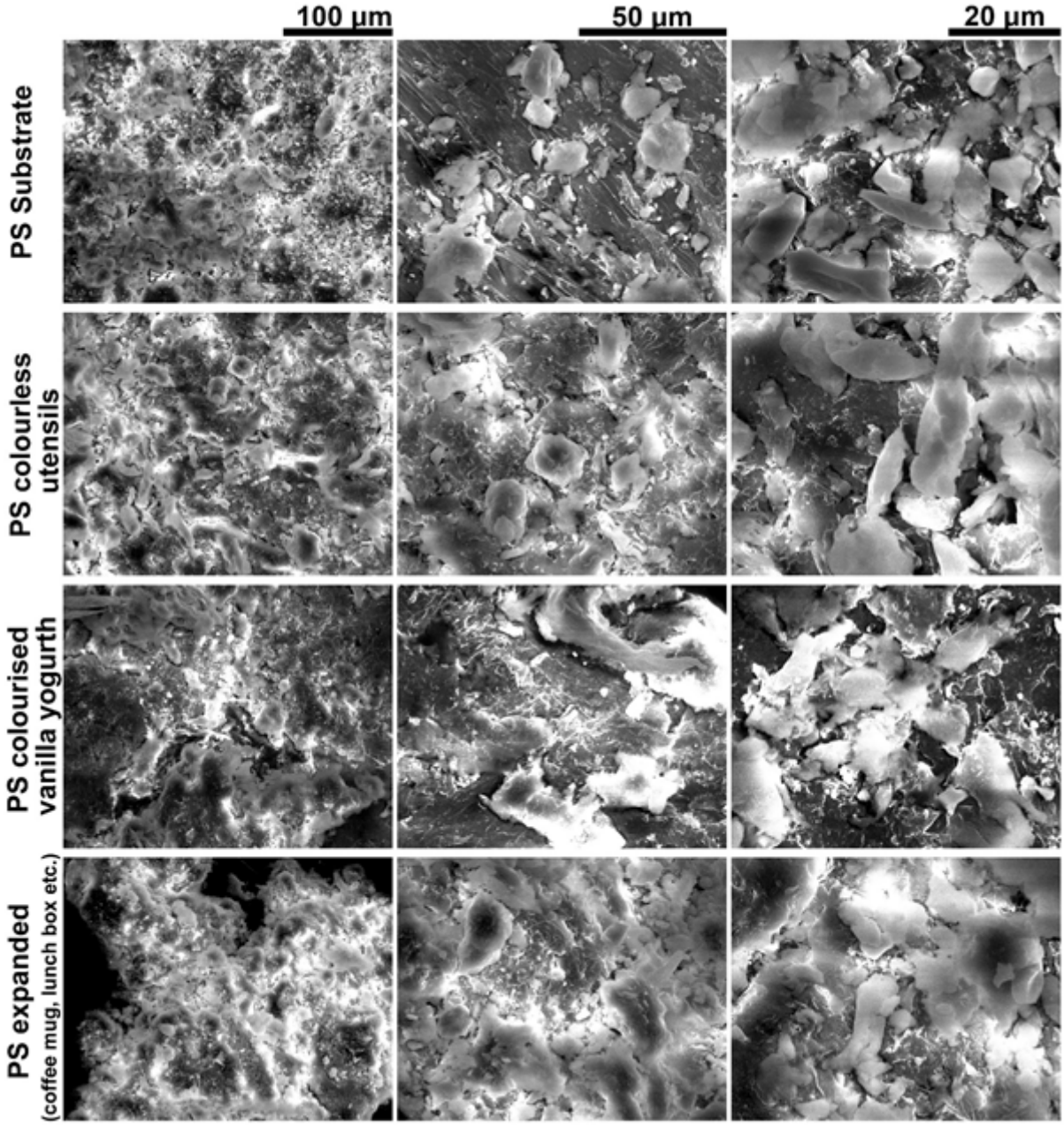


Figure 1

SEM micrographs of a few, representative examined materials after disintegration with a ball mill.

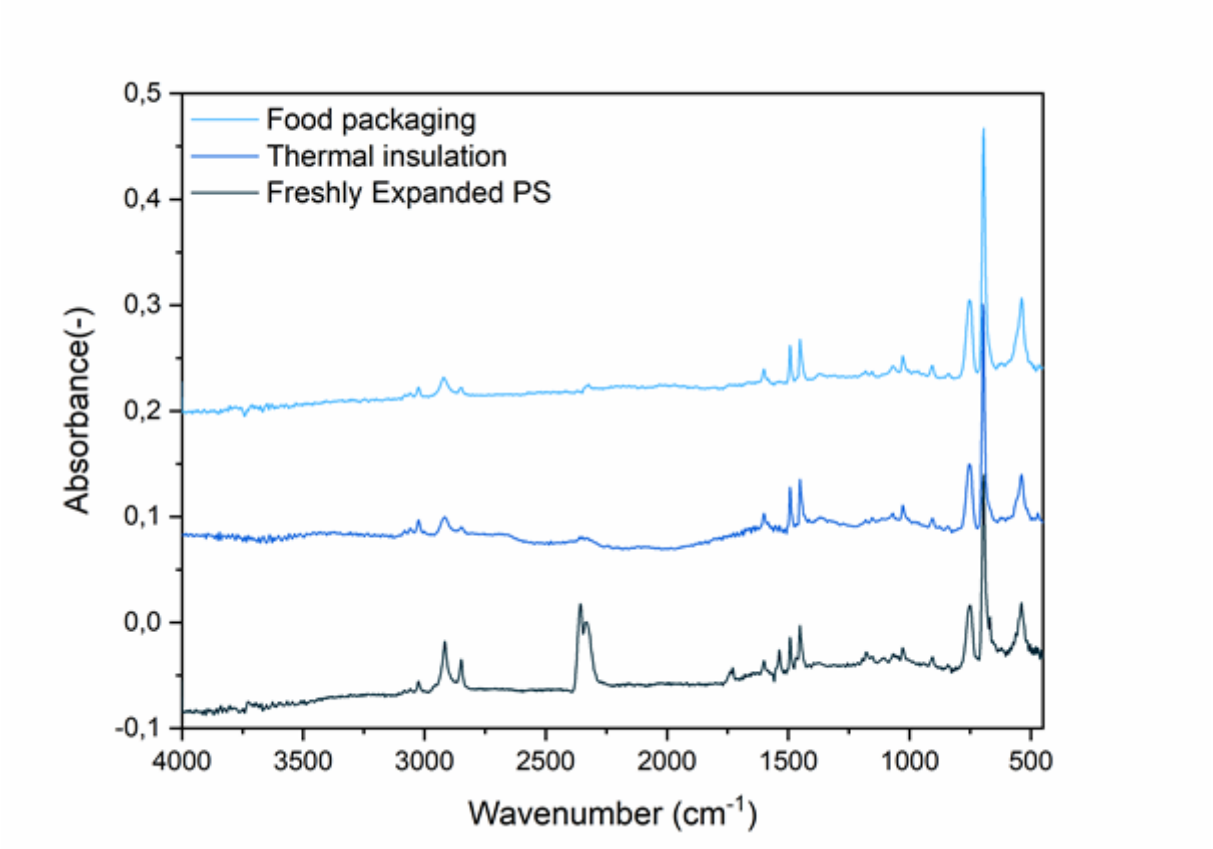


Figure 2

FT-IR spectra of PS-based products: food packaging (non-colorant), 3 years seasoned thermal insulation, and freshly expanded PS.

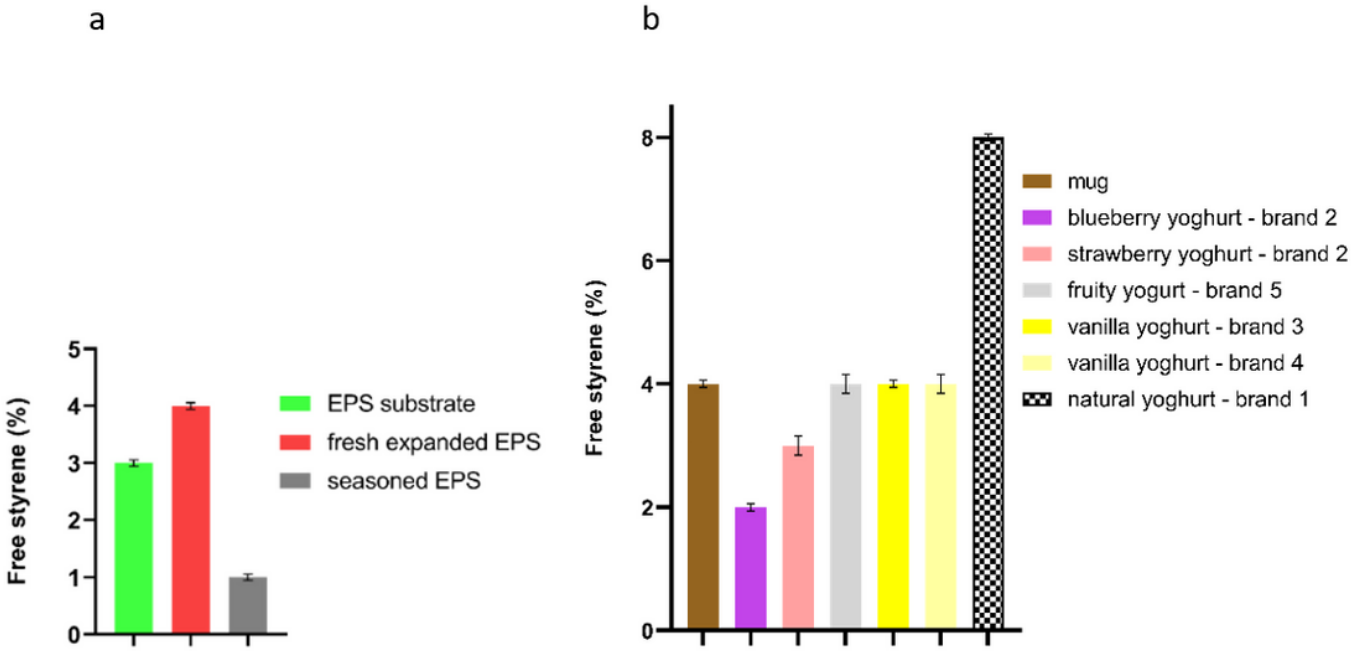


Figure 3

Free styrene content measured by Wijs methodat (A) thermal insulation material at different time of livetime of it and (B) showed sample food packagings.

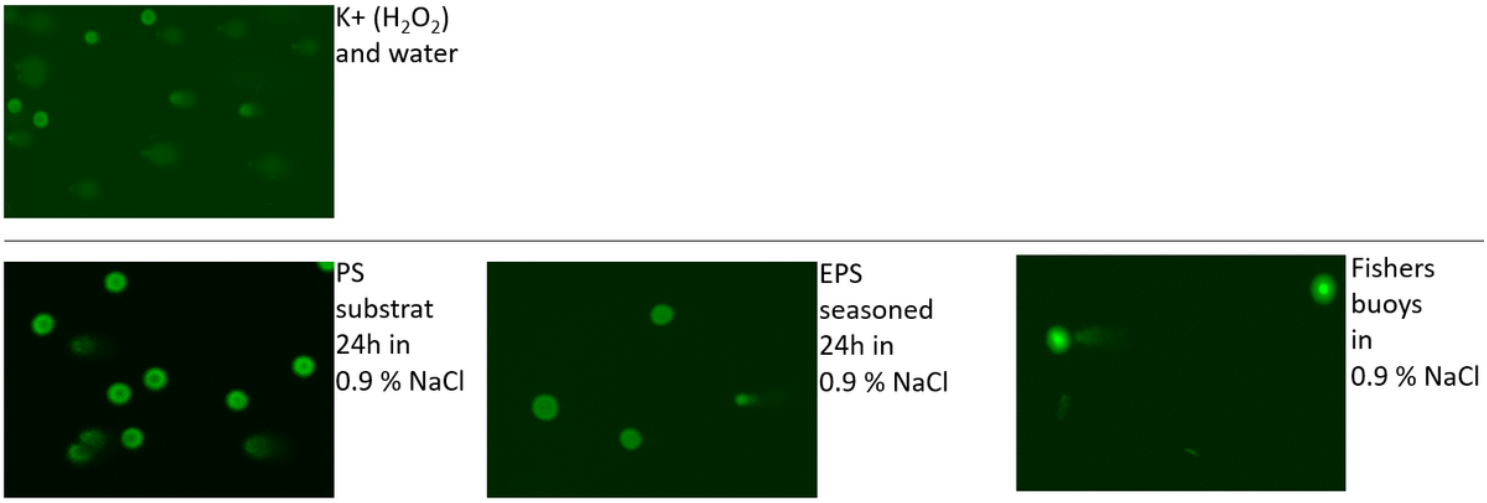
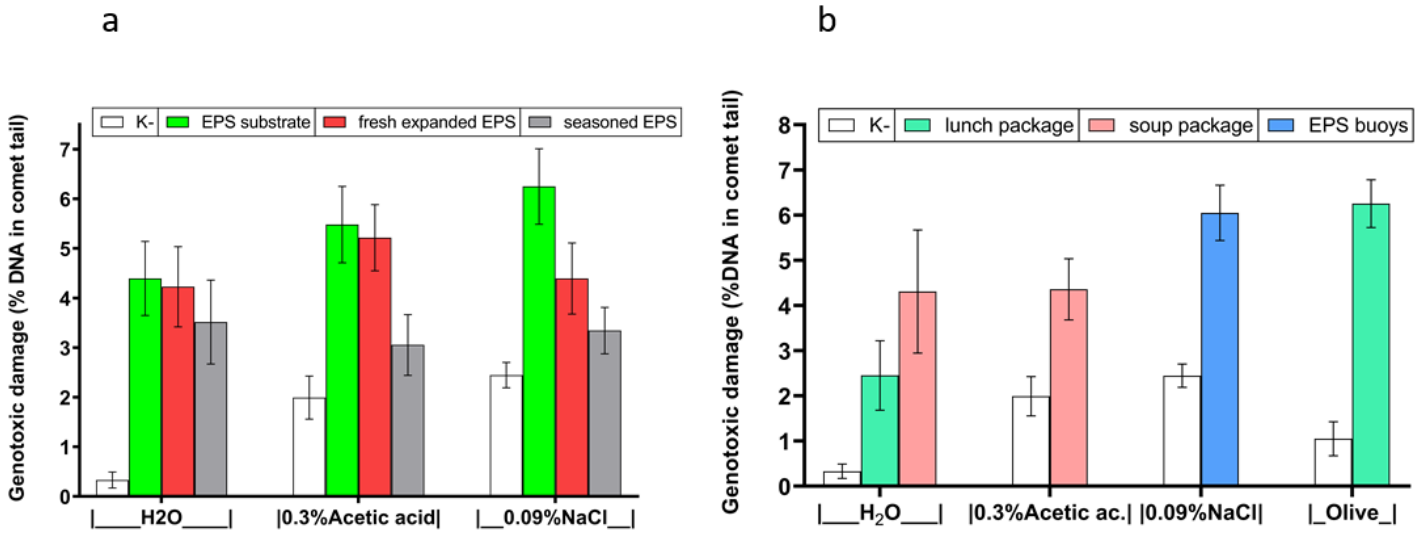


Figure 4

Images of HT29 cell nuclei stained with Sybr Green fluorescent dye.



igure 5

enotoxicity of MPs from different materials prepared in (**a**) H_2O, acetic acid and NaCl solution and in **b**) H_2O, acetic acid, NaCl solution and olive oil in HT29 cells evaluated with the aid of comet assay and xpressed as %DNA in the comet tail.

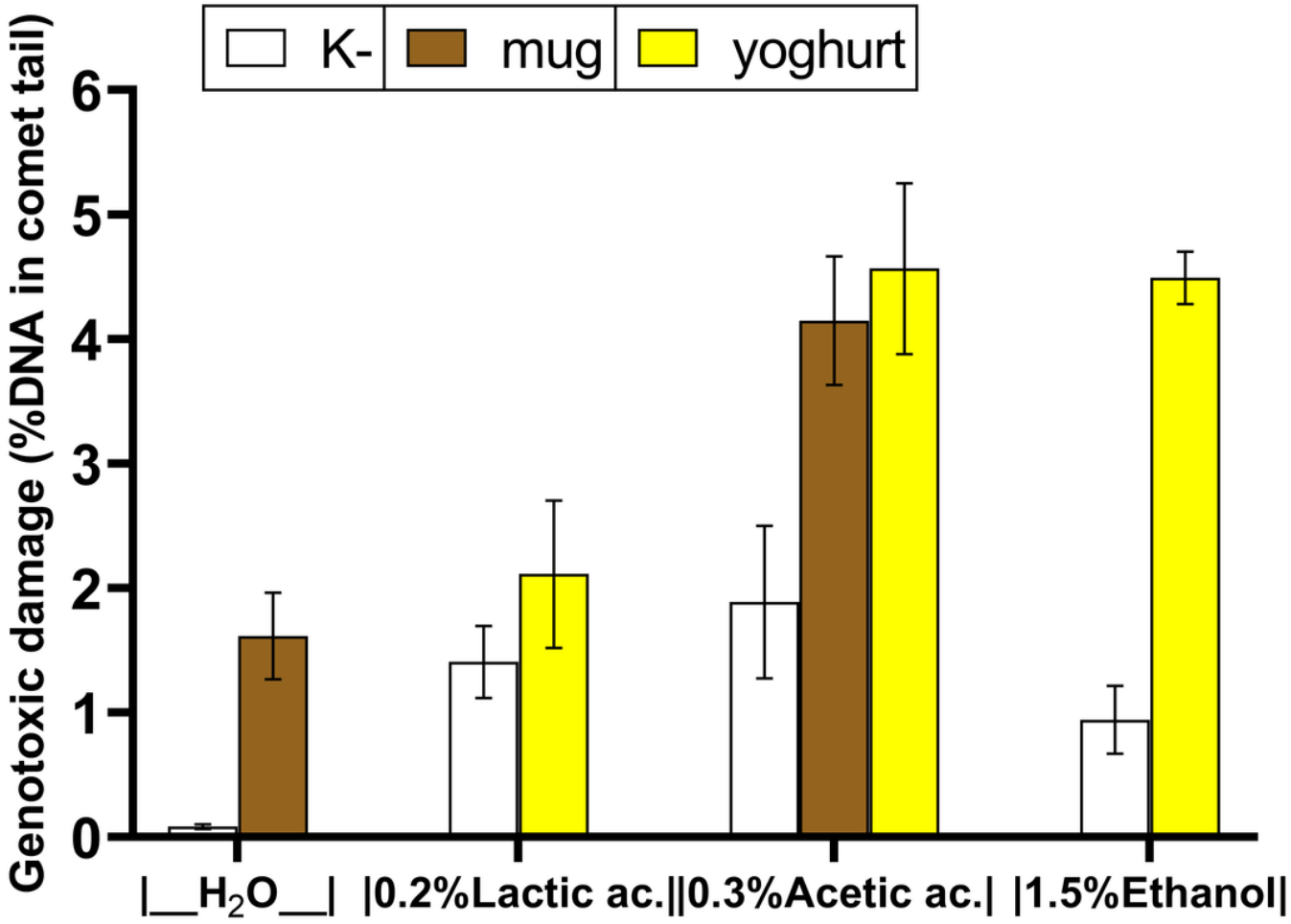


Figure 6

Genotoxicity of MPs from different materials prepared in H_2O, lactic acid, acetic acid and ethanol in HT29 cells evaluated with the aid of comet assay and expressed as %DNA in the comet tail.

Figure 7

Few samples of examined in this study PS materials.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- MSPSKPnodiffrences.pdf