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Determination of plastic polyester oligomers in real samples and their bioeffects

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Abstract

Plastics are ubiquitously, becoming part of our everyday life. Recently, the issue of human exposure to micro- and nanoplastic particles and potentially resulting toxicological consequences has been broached, triggered by the discovery of microplastics in foodstuff and dietary exposure via contaminated food and beverages. Within this EU-FORA fellowship project, a determination and quantification of plastic polyester plastics oligomers in food samples was performed to assess exposure at these categories of 'nanoplastics', evaluating them as potential contaminants or as indicators and marker compounds of the exposure to specific nanoplastics/microplastics from polyesters, such as polyethylene terephthalate (PET) and polybutylene terephthalate (PBT). UHPLC-TOF–MS/MS analysis has been set-up for 10 PET and PBT oligomers and analysis has been performed in foods and drinks. Moreover, the project focused also on the effects of these oligomers in *in vitro* and *ex vivo* experiments. These data would be combined with EFSA Comprehensive Food Consumption Database, for the exposure and risk assessment of these 'Nanoplastics'.

Keywords: plastic oligomers, nanoplastics, food, occurrence, toxicity, risk assessment

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Declarations of interest: If you wish to access the declaration of interests of any expert contributing to an EFSA scientific assessment, please contact interestmanagement@efsa.europa.eu.

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1. Introduction

Packaged food can contain regulated additives or intentionally added substances (IAS), allowed to be used for the production of plastic food contact materials (FCM). However, apart from IAS, also non-intentionally added substances (NIAS) can occur in a plastic FCM, as a result of reaction and degradation processes or due to the presence of impurities in the raw materials used for the packaging production. An important subset of the aforementioned group of substances are the oligomers, a class of substances deriving from plastic FCM that have been found in recent years to migrate in substantial amounts from the packaging to the food itself. Recently, both DG SANTE and EFSA showed an interest in this category of compounds, but real chemical, migrating and toxicological data are not available for most of these substances as no comprehensive toxicological studies have been performed. This fact has a clear impact in the determination of their safety and potential health risks.

2. Description of work programme

2.1. Aims

Legislation for microplastics and nanoplastics as contaminants in food is lacking as the knowledge is limited. There is hardly any scientific data on the exposure and the toxicity of plastic oligomers. As a result, risk assessment cannot be estimated. This EU-FORA fellowship project called 'QUantitative determination of Plastic polyester OLIGOmers in real samples' (QUPOLIGO) aimed to conduct a comprehensive framework on this topic, including data mining and systematic analysis of the literature to cover knowledge on the occurrence and potential toxicity of micro- and nanoplastics. These results would offer the base for risk assessment calculation. The project has also included experimental work for the determination of oligomers in *in vivo*, *ex vivo* samples and foods. A particular focus have been given to three categories of plastic FCM oligomers, which include a regulated group of cyclic oligomers from polystyrene (PS), polyethylene terephthalate (PET) and polybutylene terephthalate (PBT). For PS oligomers, EFSA published a Scientific Opinion regarding Assessment of the impact of the IARC Monograph on the safety of the styrene substance (FCM No 193) and its respective oligomers in plastic FCM, for which no genotoxicity data nor mechanistic data or comparative toxicokinetics and analysis of species differences are existing at the moment. All PS oligomers are considered NIAS. Regarding PET, these cyclic oligomers can potentially migrate from plastic FCM to foods. Typical examples of cyclic oligomers found frequently are those resulting from reactions between adipic acid (AA) and phthalic acid (PA) with diethylene glycol (DEG) from multilayer materials, as well as terephthalic acid (TPA) with ethylene glycol (EG). However, no data on real food samples exist nor potential toxicological effects. Furthermore, it shall not be omitted that at the moment only PET is allowed to be mechanically recycled at EU level, where EFSA deals with a large number of FCM recycling applications to be assessed. All PET cyclic oligomers are considered as NIAS. Finally, PBT oligomers (FCM 885) are regulated and allowed to be used with certain limitations for the production of plastic FCM, according to European Commission Regulation No 10/2011. This mixture of PBT cyclic oligomers consists of the dimer, trimer, tetramer and pentamer at a specific ratio and are considered as IAS. No data are available for the concentrations of these compounds into real foods and analysis methods to detect them are missing.

2.2. Activities/methods

2.2.1. Revision of available data

A comprehensive literature search has been performed in order to retrieve peer-reviewed articles referring to the presence of the selected oligomers in foods, including beverages, as well as for their toxicity, either *in vivo* or *in vitro*. Scientific databases such as 'PubMed', 'Scopus', 'Google Scholar' and 'Web of Science' were searched using various keyword combinations (e.g. 'polymer', 'oligomers', 'cyclic oligomers', 'PS', 'PET', 'PBT', 'food ', 'toxicity', 'in vivo', 'in vitro'). Then, a more focused research has been performed to restrict the field with a combination of words related to the effects of food contact materials on children ('risk assessment', 'toxicity', 'analysis and occurrence', 'oligomers', 'children').

2.2.2. Analytical work/evaluation of concentrations

The scope of this part of the fellowship was to train the applicant by participation in research through the development and validation of methods for analysis of the oligomers of interest in various specimens. Work has been built upon the development of a method for ultra-high performance liquid chromatography-time-of-flight tandem mass spectrometry (UHPLC-TOF-MS/MS) analysis for the determination of oligomers in real samples. Methods for sample preparation developed in the lab have been set up (Diamantidou et al., 2022; Diamantidou et al., 2023). The method determined the following monomers: (1) PET dimer, (2) PET trimer d12 (internal standard), (3) PET trimer, (4) PBT dimer, (5) PET tetramer, (6) PET pentamer, (7) PBT trimer, (8) PBT tetramer with high sensitivity (limit of quantifications in the area of 5–12 μg L^{-1}). A calibration and preparation of solutions in standard mixtures has been done. Stock solutions of the PET dimer and the isotope internal standard were prepared at a concentration of 5000 mg L^{-1} in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) whereas PET trimer, tetramer and pentamer stock solutions were at 2,500 mg L^{-1}. PBT mix solution in HFIP was at a concentration of 20 mg L^{-1}, in HFIP as solvent. A multi-component solution (2.5 mg L^{-1}) containing equal concentrations of every analyte was prepared by mixing the appropriate volumes of the stock solutions. Serial dilutions of this mix solution followed in amber vials with ethanol:H_2O 50:50 (v/v), to prepare working standard mixtures at 10 concentrations (5, 10, 25, 50 100, 250, 500, 750, 1,000, 2,500 μg L^{-1}). Stock solutions and working standards were both kept at −20°C. Calibration curves were constructed by plotting the means of ratios of the compound peak areas to the internal standard peak areas against concentrations of the analytes. Four different 0.5-L PET bottles intended for contact with beverages and edible oils were supplied from local market. Two of them were from 100% virgin PET (vPET) and the other two from 100% recycled PET (rPET). In the first test, the migration experiment was performed according to the Reg. EU No 10/2011 (European Commission, 2011). For water samples, compliance testing has been carried out using ethanol (20%) in water. Then, both the vPET and the rPET plastic bottles containing the food simulants were stored in an incubator at a temperature of 60°C for 10 days. Each sample volume was adjusted to 400 mL and two replicates were prepared. Test time and temperature were selected to cover a storage period of more than 6 months at room temperature or below, mimicking potential household conditions. For the migration measurements in tap water, time and temperature storage conditions representative of household conditions/real life were selected. Thus, in this second migration experiment, PET bottles were filled with 400 mL of 'blank' tap water sample. Three replicates for each type of PET bottle (virgin and recycled) were prepared and were stored for 12 months at room temperature (~ 20°C). Samples were collected at three time points (2, 6 and 12 months) and analysed. An UHPLC system (Bruker, Germany) was used for chromatographic separations of samples using a Waters BEH C18 (150 × 2.1 mm, 1.7 μm) analytical column, protected by a UPLC BEH C18 (5 × 2.1 mm, 1.8 μm) VanGuard pre-column. The method is fully described in Diamantidou et al. (2022). Briefly, the mobile phase consisted of solvent A: H_2O with 0.1% formic acid and solvent B: ACN with 0.1% formic acid at a flow rate of 0.3 mL/min. The elution was performed in a 12-min gradient as follows: 0–1 min: 50–70% B; 1–12 min: held to 90% B. The composition was returned to the initial (50% B) in 0.1 min. An equilibration time of 4 min was set for the column before the next injection. The analytical column was temperature-controlled at 60 °C and the injection volume was 5 μL. The system was operated by Compass HyStar 5.1 software (Bruker, Germany) and was hyphenated to a timsTOF mass spectrometer (Bruker, Germany) operating in positive ionisation mode at a 3.5 kV capillary voltage. The source operated at 300°C and nitrogen was used as drying (10.0 L/min) and nebulising gas (2.0 Bar). The Funnel 1 RF, Multipole RF, Deflection Delta and Collision RF were set at 200 V, 200 Vpp, 50 V and 700 Vpp, respectively. The acquisition mode was set at full scan acquiring data over the range of 300–1,000 m/z at a rate of 3 spectra/s. For individual recalibration of the chromatograms, sodium formate solution was injected before every analysis from 0.1 to 0.3 min. Data Analysis 5.3 software was used for data handling (Bruker, Germany).

2.2.3. *In vitro* experiments

The potential of oligomers for cellular viability has been assessed. For this purpose, Caco-2 cell line has been used to mimic small intestine conditions. Based on the results of experiment described in the previous paragraph, three PBT oligomer concentrations (no oligomers as control; low concentration; high concentration) has been applied. All experiments have been performed in triplicate. The cells were exposed and incubated for 24 and 48 h (Figures 1 and 2).

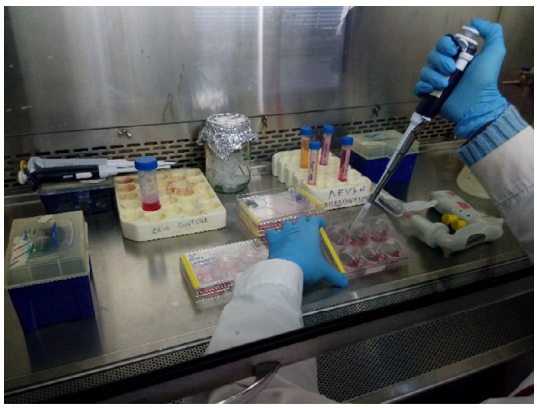

Figure 1: Preparation of cell cultures for the experiments

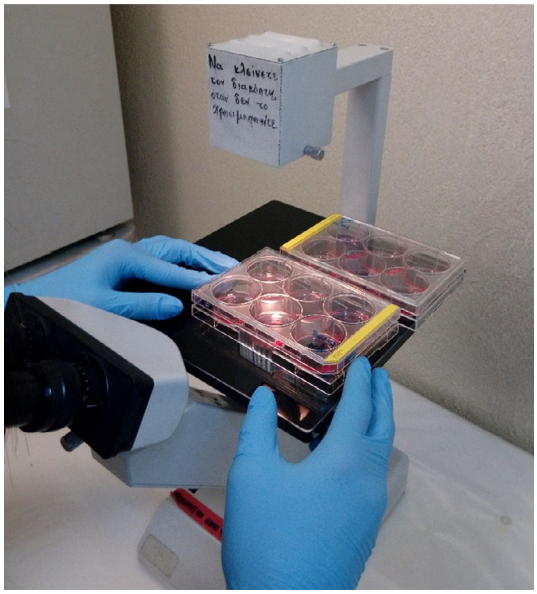

Figure 2: Part of the procedure to check the confluency of cells in each well/plate with the invertoscope

After 24 h and 48 h, cells were tested for apoptosis and cytotoxicity level.

Apoptosis were checked by using Fluoroisothiocyanate Annexin V Apoptosis Detection Kit (BioLegend; Cat# 640914). Annexin V (or Annexin A5) is a member of the annexin family of intracellular proteins that binds to phosphatidylserine in a calcium-dependent manner. Phosphatidylserine is normally only found on the intracellular leaflet of the plasma membrane in healthy cells, but during early apoptosis, membrane asymmetry is lost and phosphatidylserine translocates to the external leaflet. Fluorochrome-labelled Annexin V can then be used to specifically target and identify apoptotic cells. To help distinguish between the necrotic and apoptotic cells, propidium iodide solution has been used. Early apoptotic cells will exclude phosphatidylserine, while late stage apoptotic cells stained positively, due to the passage of the dyes into the nucleus where they bind to DNA. When excited by 488 nm laser light, propidium iodide, a fluorescent dye that binds to DNA, can be detected and it is commonly used in evaluation of cell viability or DNA content in cell cycle analysis by flow cytometry.

Cytotoxicity was tested by lactate dehydrogenase assay (Biolegend, Cat# 426401). The determination of cytotoxicity has been performed by measuring lactate dehydrogenase activity released from damaged cells. Lactate dehydrogenase catalyses dehydrogenation of lactate to pyruvate thereby reducing NAD^+ to NADH. NADH reduces water-soluble tetrazolium salt in the presence of an electron mediator to produce an orange formazan dye.

2.2.4. *Ex vivo* or permeability experiments

Rat gut sac is a paradigm widely employed for determination of absorption kinetics of drugs along with evaluation of effects of absorption enhancers. In this case, the potential of oligomers for permeation through the membrane tract (8 cm) has been evaluated after 15, 30, 60, 90 and 120 min from the exposition to different oligomer concentrations at 37°C. The same concentrations of the *in vivo* experiments have been adopted (Figures 3 and 4).

Figure 3: Preparation of the rat gut for the permeability experiment

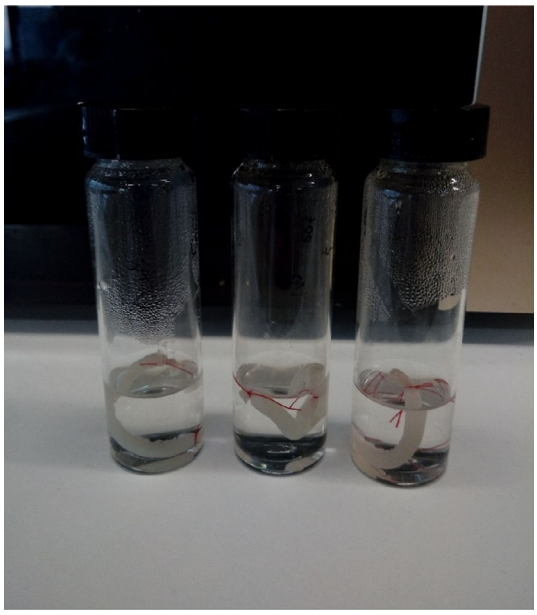

Figure 4: *Ex vivo* permeability study evaluated using the non-everted gut sac method

Then, the released oligomers out the lumen have been carried out by using UHPLC-TOF–MS/MS.

2.2.5. Training in risk assessment

Risk assessment training had the aim to train the applicant in calculating the risk assessment of target oligomers. The applicant has attended training activities from EFSA. In addition, the host has provided some training activities to try to calculate the risk assessment of PET, PS and PBT oligomers based on the concentrations that will be found in the experiments described in the above paragraphs. In the future, all the results of these activities are going to be maximised by knowledge transfer in participating to workshops, conferences, panels and working groups meetings at the hosting site as well as at the EU level (EFSA conferences and international workshops) and at fellow sending organisation.

3. Conclusion

Overall, the work programme allowed the fellow to gain knowledge on the analysis of plastic oligomers and their effects. On the one hand, an analysis and evaluation of available data on the occurrence of NIAS in food contact materials and their toxicological effects on children has been performed. On the other hand, the fellow gained knowledge in practical research work, with respect to both oligomer detection and *in vitro* and *ex vivo* toxicity assessment. The work on plastic oligomers was embedded into the overall context of food risk assessment and opportunities for scientific networking and collaboration. Results of the fellow's project will become part of scientific papers to be published in a peer-reviewed scientific journal.

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Abbreviations

AA	adipic acid
BIOMIC	Centre for BIOanalysis and OMICs
DEG	diethylene glycol
EG	ethylene glycol
EU-FORA	European Union Food Risk Assessment fellowship programme
FCM	food contact materials
HFIP	1,1,1,3,3,3-hexafluoro-2-propanol
HILIC	hydrophilic interaction liquid chromatography
IARC	International Agency for Research on Cancer
IAS	intentionally added substances
NIAS	non-intentionally added substances
PA	phthalic acid
PBT	polybutylene terephthalate
PET	polyethylene terephthalate
PS	polystyrene
rPET	recycled polyethylene terephthalate
RPLC	reversed-phase liquid chromatography
TPA	terephthalic acid
UHPLC-TOF–MS/MS	ultra-high performance liquid chromatography-time-of-flight tandem mass spectrometry
vPET	virgin polyethylene terephthalate