# Migration studies and toxicity evaluation of cyclic polyesters oligomers from food packaging adhesives

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## Abstract

Multilayer materials used in food packaging are commonly manufactured with a polyurethane adhesive layer in its structure that may contain cyclic esters oligomers as potential migrants. However, little is known about their toxicity. In this work, two cyclic esters of polyurethane are evaluated in migration from 20 multilayer packaging samples. They were composed by adipic acid (AA), diethylene glycol (DEG) and isophthalic acid (IPA) and their structure was AA-DEG and AA-DEG-IPA-DEG. The concentration of these compounds in migration exceeded the maximum level established by Regulation EU/10/2011 (10 ng $g^{-1}$). Bioaccessibility of both compounds was evaluated by studying gastric and intestinal digestion. The studies showed that the concentration of the compounds decreased during digestion and that their hydrolysed molecules increased. Furthermore, endocrine activity in vitro assays were performed. A weak androgen receptor antagonism was identified, whereas no arylhydrocarbon receptor activity or binding to the thyroid hormone transport protein was found.

***Keywords:*** *oligomers, migration, NIAS, polyurethane adhesive, food packaging, bioaccesibility, endocrine activity*

## 1. Introduction

Food contact materials (FCMs) protect food from external contamination and preserve the nutritional value as well as the physical and sensory quality of food. However, it is important to control the migration of compounds from packaging materials to foods, as it may lead to the transfer of unwanted substances that can make food less safe for consumption or that may alter its sensory and nutritional characteristics. It is necessary, therefore, to identify the compounds that are present in the different packaging materials and that can be potential migrants (Wrona & Nerín, 2019). Substances in FCM can be intentionally or non-intentionally added (IAS and NIAS). NIAS are difficult to control, as they are often not chemically well characterized and are present at low concentration levels. This complicates its identification and therefore, advanced techniques with high sensitivity and resolution are needed (Margarita Aznar, Ubeda, Dreolin, & Nerín, 2019; Hoppe, de Voogt, & Franz, 2016; Nerin, Alfaro, Aznar, & Domeño, 2013; Pietropaolo, Albenga, Gosetti, Toson, Koster, Marin-Kuan, et al., 2018).

In the case of multilayer packaging materials, where the material is made of multiple polymer layers bonded by adhesives, migration can occur not only from the material that is in direct contact with food, but also from internal layers of the material including the adhesives. This process is due to diffusion and partition processes of the compounds between the different layers (Margarita Aznar, Vera, Canellas, Nerín, Mercea, & Störmer, 2011; Tehrany & Desobry, 2004). Due to its thermal stable temperature properties, flexibility, durability and impact resistance, polyurethane (PU) is the most commonly used adhesive for flexible multilayer structures (Heath & Cooper, 2013). PU adhesives are also used in other applications such as in the assembly of shoes, automotive interiors, windshield bonding or textile laminates (Engels, Pirkl, Albers, Albach, Krause, Hoffmann, et al., 2013). Therefore, there can be different potential exposure sources of these compounds.

PU adhesive synthesis is a reaction between di-isocyanates and linear polyester compounds, where the latter are produced by polycondensation reaction between polyols (ethylene glycol, EG; diethylene glycol, DEG; 1,4-butanediol, BD; neopentyl glycol, NPG; 1,6-hexanediol, HD) and aliphatic or aromatic carboxylic acids (adipic acid, AA; isophthalic acid, IPA). When the last reaction does not proceed under equilibrium conditions, it favors the formation of short chain cyclic polyesters, so-called lactones, in addition to linear polyesters (Shrikhande, 2012). These cyclic esters can also be considered oligomers as they are formed by several monomer units. The formation of cyclic esters is undesirable from an industrial point of view as they can impair the physical properties of the material (Eceiza, Martin, de la Caba, Kortaberria, Gabilondo, Corcuera, et al., 2008; Shrikhande, 2012; Zhang, 2014). Furthermore, from a food packaging perspective, these

unwanted by-products are considered NIAS and, as demonstrated previously (Félix, Isella, Bosetti, & Nerín, 2012; Gómez Ramos, Lozano, & Fernández-Alba, 2019; Nerin, Alfaro, Aznar, & Domeño, 2013; Ubeda, Aznar, & Nerín, 2018; Úbeda, Aznar, Vera, Nerín, Henríquez, Taborda, et al., 2017; Zhang, Kenion, Bankmann, Mezouari, & Hartman, 2018) have a high migration potential. Migration of theses oligomers could be seen as microplastics coming from plastic FCMs (Ubeda, Aznar, Alfaro, & Nerín, 2019). As they are NIAS, they are not included in any database and often commercial standards are not available, making identification and confirmation a difficult process. Other byproducts coming from PU are the primary aromatic amines (PAAs) which are possibly carcinogenic to humans (Campanella, Ghaani, Quetti, & Farris, 2015).

There is no specific European legislation for food packaging adhesives and its components, though some countries such as Switzerland have a national legislation (Swiss-Confederation, 2013). However, when PU adhesive are used in the manufacture of multilayer plastic for FCM they are controlled by Regulations 1935/2004/EC (EC, 2004) and 10/2011/EU (EC, 2011). The Regulation states that FCM components must not be transferred into food in quantities that may harm human health. The oligomers are not specified in the Regulation 10/2011/EU (EC, 2011), thus a limit of migration to food simulants of 10 ng $g^{-1}$should not be exceeded.

There is little information on the hazards of oligomers. This is partially due to the lack of commercial standards necessary for toxicological testing. It has often been assumed that oligomers have the same toxicity as their starting monomers and that they should therefore be covered by their toxicological evaluation (Grob, Camus, Gontard, Hoellinger, Joly, Macherey, et al., 2010; Nelson, Patton, Arvidson, Lee, & Twaroski, 2011). However, it is evident that reaction products can have different properties. According to EFSA (EFSA, 2008), when the polymer is formed by the polymerization of an approved monomer, its lack of genotoxicity is established by the data on the monomer, and no requirement for experimental data on the polymer itself are needed such as for cyclic butylene terephthalate (EFSA, 2009). In some cases, the same toxicity results of monomers and their oligomers have been demonstrated, such as for oligomers of halocarbon 3.1 oil and chlorotrifluoroethylene trimer acid (Nelson, Patton, Arvidson, Lee, & Twaroski, 2011). In contrast, it has been demonstrated in other cases that the toxicological profile of the reaction products and starting substances differed, such as the oligomers of styrene (Gelbke, Banton, Block, Dawkins, Leibold, Pemberton, et al., 2018). Thus, it is important to test the toxic potential, not only of the starting material, but also of the present oligomers. Initially, these tests can be done by in vitro examinations.

The safety evaluation from the Office of Food Additive Safety (OFAS) states that oligomeric materials with a molecular weight below 1000 Da are important from a toxicological point of view as they could migrate into food and be absorbed in the gut (Nelson, Patton, Arvidson, Lee, & Twaroski, 2011). Hence, it is crucial to assess the safety of those oligomers with lower molecular weights. However, as far as the authors know, the toxicological properties are not well characterized.

In addition, foodstuff undergoes a series of processes before being absorbed into the body, such as gastric and intestinal digestions. These processes might change the concentrations of substances available to be absorbed and could even lead to the formation of new compounds. These changes may have implications for the final toxicity. Thus, it is important to study the bioaccessibility as well as the gastrointestinal degradation of the migrant compound to enhance the understanding of the chemical composition of the fraction available for absorption (M. Aznar, Gómez-Estaca, Vélez, Devesa, & Nerín, 2013).

Exogenous compounds such as endocrine disrupting chemicals (EDCs) are of special interest because they mimic, block or in other ways alter the activities of endogenous hormones. In vitro assays have been developed for a wide range of toxicological effects including induction of cytochrome P450 enzymes, androgenic activity and thyroid disruption. The binding or blocking of steroid hormone receptors like the androgen (AR) receptor by chemicals has been a significant focus for assessment of endocrine disruption potential as this receptor has got a pivotal role in development of male reproductive health (Schwartz, Christiansen, Vinggaard, Axelstad, Hass, & Svingen, 2019). Increasing attention is now being given to the ability of chemicals to disrupt the thyroid hormones system, which play an important role in ensuring normal development of the embryonic brain (Duntas & Stathatos, 2015). Another important assay is the aryl hydrocarbon receptor (AhR) assay that – when activated – leads to increased metabolism of chemicals, drugs, and hormones and which also plays an important role in our immune defense (Esser & Rannug, 2015).

In this study, the objective was to investigate migration of two cyclic esters from multilayer packaging material based on PU adhesives, as well as to evaluate their bioaccessibility to the body. The potential formation of new compounds during gastrointestinal digestion was also evaluated. Furthermore, the in vitro endocrine disruptive potential of both compounds was studied in assays covering androgen receptor and aryl hydrocarbon receptor activity, as well as binding to transthyretin – an important transport protein of thyroid hormones.

## 2. Materials and methods

### 2.1 Test chemical

Two cyclic ester oligomers, AA-DEG and AA-DEG-IPA-DEG, composed of diethylene glycol (DEG), adipic acid (AA) and isophthalic acid (IPA) were tested. Test substances were chemically synthesized and their structures and purity were confirmed by NMR at the University of Zaragoza. The high resolution mass spectra of these compounds will be described in the *Results* section.

### 2.2 Samples

Twenty multilayer plastic materials mainly intended for FCM and the storage of biological fluids were tested (samples code: 1S-20S). Polyurethane was used as adhesive in the manufacture of all evaluated samples. The materials contained a combination of aluminium (Al), polyethylene terephthalate (PET), polyamide (PA), polypropylene (PP) and polyethylene (PE) and had different thickness. They were supplied by different manufacturing companies and are described in Table 1.

### 2.3 Migration test

For the migration experiments, multilayer materials were cut (10 x 10 $cm^2$), folded in half and thermo-sealed. The internal surface of the bags was 0.64 $dm^2$. Afterwards, they were filled with different simulants. The simulants used, as well as the temperatures and times of the migration experiments were selected depending on the intended use of the material and according to EU/10/2011 (EC, 2011). Ultrapure water (Milli-Q Ultramatric Wasserlab GR 216071, Madrid, Spain) and ethanol 10 % were used as aqueous simulants and ethanol 95 % (Panreac, Barcelona, Spain) as fat simulant. Water was used when the materials were intended for biological fluids. When samples were intended for food contact, 10% ethanol was selected for food with hydrophilic character and 95% ethanol for fat and dry food.

EU/10/2011 (EC, 2011) established that for contact times above 30 days at room temperature, materials should be tested in an accelerated test at 60 °C for a maximum of 10 days. For contact times longer than 2 days at room temperature, three days at 40ºC was selected. For pasteurized materials, the conditions were different. In this case, bags were introduced in a stainless steel extraction cells, completing the cell space with water and maintaining the assembly for 30 min at 121 °C. This way, the ethanol is kept in liquid phase during the assay, due to the pressure exerted under these conditions by the water inside the cell. In the case of biological samples, tests were performed at 40ºC for 3 days on the basis of its use.

158 Although the materials had dissimilar end use, the migration concentrations were corrected to 6 $dm^2$
159 of packaging material per 1 kg simulant, in accordance with European Regulation 10/2011 (EC,
160 2011) to compare results. Three replicates of every test were analysed. Samples were analysed by
161 UPLC-QTOF.

162 **2.4 Digestion assays**

163 The protocol was prepared according to 2008 EFSA guide (EFSA, 2008). The experiments were
164 carried out in three independent replicates and analysed by UPLC-QqQ (MRM mode) and UPLC-
165 QTOF.

166 **2.4.1 Gastric digestion**

167 Gastric simulant was 0.07 M HCl (35 %, Panreac). The pH of the solution was 1.2 ± 0.1.

168 An aliquot of 100 µL of cyclic ester (100 µg/g water) was added to 10 mL of gastric simulant (final
169 concentration 1 µg/g) and afterwards heated at 37ºC. This solution was maintained with agitation at
170 37ºC for 4 h. During digestion, aliquots of 1 mL were taken at 4 different times ($t_0$, $t_{1h}$, $t_{2h}$ and $t_{4h}$)
171 and neutralized with 250 µL 0.02M sodium hydroxide (NaOH) (1M, Panreac) at pH 6.

172 **2.4.2 Intestinal digestion**

173 Intestinal simulant was carried out with pancreatin from porcine pancreas (Sigma Aldrich)
174 according to 2008 EFSA Guide (EFSA, 2008).

175 For its preparation, 6.8 g of potassium dihydrogen orthophosphate ($KH_2PO_4$) (Pro Analyse Merck)
176 was dissolved in 250 mL water and transferred to a 1 L volumetric flask to which 190 mL 0.2 M
177 NaOH and 400 mL water were added and mixed briefly. Then, an amount of 10 g of pancreatin
178 extract was introduced into a 250 mL beaker with little water to make a homogenous paste. After
179 this, the paste was gradually diluted with small portions of water, stirring well after each dilution to
180 give approximately 150 mL of a lump-free solution. The solution was transferred to the 1 L
181 volumetric flask where 0.5 g of sodium taurocholate (Sigma-Aldrich) were added and shaken. Then,
182 water was added leaving space to adjust pH to 7.5 ± 0.1 with 0.2 M NaOH.

183 Digestion assay was carried out adding 50 µL of 100 µg $g^{-1}$ of cyclic ester in water to 10 mL of
184 intestinal simulant previously tempered at 37ºC and (500 ng $g^{-1}$ final concentration). This
185 dissolution was maintained at 37ºC with constant agitation. During digestion, aliquots of 1 mL were
186 taken and evaluated at 4 different time points ($t_0$, $t_{1h}$, $t_{2h}$ and $t_{4h}$). In order to precipitate the proteins
187 present in the aliquot, 1 mL of 20 % (w) trichloroacetic acid (TCA) (Sigma-Aldrich) was added to

each aliquot and then cooled on ice bath for 30 min. Successively, the solutions were centrifuged at 8000 rpm for 15 min and 1 mL of the supernatant was filtered (PET 0.22µm) and transferred to a vial with 250 µL of 0.02 M NaOH to adjust to neutral pH.

In order to check if the addition of TCA could degrade the cyclic esters, 500 µL of cyclic ester were mixed with 500 µL of TCA and 250 µL of 0.02M NaOH and the results were compared to the cyclic esters without TCA addition. The signals were similar in both experiments and therefore it was concluded that TCA did not hydrolyse the cyclic ester.

### 2.5 Instrumentation and conditions

#### 2.5.1 Ultra-performance liquid chromatography analysis (UPLC)

Chromatography was performed using an Acquity™ system with a UPLC BEH C18 column of 2.1 mm x 100 mm and 1.7 µm particle size supplied by Waters (Milford, MA, USA). The column temperature was 40 ºC and the column flow was 0.3 mL/min. The sample injection volume was 10 µL (QTOF) and 5 µL (QqQ). Mobile phases were water (phase A) and methanol (phase B) with 0.1% formic acid. Chromatography started at 98/10 phase A/phase B, changed to 0/100 in 7 minutes.

#### 2.5.2 MS-QTOF conditions

MS-QTOF analysis was performed in a Xevo G2 mass spectrometer supplied by Waters (Milford, MA, USA). The detector consisted of an API source (atmospheric pressure ionization) with an electrospray ionization (ESI). The electrospray probe was used in positive (ESI+) and negative (ESI-), both in sensitivity mode. The accuracy and reproducibility of all the analyses were guaranteed by use of a LockSpray™. The mass range considered was from 50 to 1200 Da. The capillary voltage was 2.5 kV, the cone voltage was 30 V and the source temperature was 120 ºC. The desolvation gas temperature and flow were 450 ºC and 550 L $h^{-1}$ respectively. The cone gas flow was 20 L $h^{-1}$

The acquisition was carried out in $MS^E$ mode with two functions; acquiring at low-energy (function 1) to obtain information about the precursor ion and at high energy (function 2) to provide information about fragment ions. The collision ramp energy was from 15 to 30 V.

MassLynx v.4.1 software (Waters, Milford MA, USA) was used to analyse the samples.

### 2.5.3 MS-QqQ conditions

MS-QqQ analysis was performed in TQ mass spectrometer from Waters (Milford, MA, USA). The UPLC system was coupled with an ESI probe to the QqQ. The electrospray probe was used in positive (ESI+) and acquisition was performed in MRM (multiple reaction monitoring) mode. The parameters used were as follow: capillary voltage was 3.5 kV, source temperature was 150ºC, desolvation temperature 450ºC, cone gas flow 60 $Lh^{-1}$, and desolvation gas flow 600 $Lh^{-1}$.

The parent ion was 217.1 [MH+] for AA-DEG and the mass transitions 217.1 → 173.1, 217.1 → 155.1 and 217.1 → 111.05 were monitored. The parent ion used for AA-DEG-IPA-DEG was 453.18 [MH+] and mass transitions 453.18 → 237.08, 453.18 → 193.05 and 453.18 → 155.07 were monitored. Cone and collision voltages were optimized from 20 to 70V. Finally, 30V cone voltage and 20V were selected as optimum values for both compounds.

Monomers were measured under the same conditions but in negative (ESI-) mode for AA and IPA and positive mode (ESI+) for DEG. In both cases the analysis was performed in SIR mode (single ion recording), being the ions monitored:145.05 $[M-H]^-$, 165.02 $[M-H]^-$ and 129.3 $[MNa]^+$ for AA, IPA and DEG respectively.

MassLynx v.4.1 and QuanLynx software were used to analyse the samples.

## 2.6 In vitro endocrine activity

Stock solutions of AA-DEG and AA-DEG-IPA-DEG of 100 mM were prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Copenhagen, Denmark).

### 2.6.1 Androgen receptor (AR) reporter gene assay

The potential of the test substances to affect AR activity was tested in an AR reporter gene assay using a stably transfected AR-EcoScreen™ cell line based on Chinese hamster ovary cell line (CHO). The protocol was essentially according to the OECD test guideline (Guidelines for the Testing of Chemicals, 2016). The cells contain three stably transfected constructs: a human androgen receptor expression construct, a firefly luciferase reporter construct with an androgen response element, and a renilla luciferase reporter construct. The latter is used to examine compromised cell viability.

Cells were cultured in Phenol Red Free Gibco® Dulbecco's Modified Eagle Medium F-12 Nutrient Mixture (D-MEM/F-12) supplemented with 5% fetal bovine serum (FBS), 200 μg/mL zeonin, 100

µg/mL hygromycin, 100 units/mL penicillin and 100 µg/mL streptomycin. All medium components were supplied by InvitrogenTM, Life TechnologiesTM (Carlsbad, California, USA).

Cells were seeded in white 96-well plates (Perkin Elmer) to a final concentration of 9 x $10^3$ cells/well in assay medium (Phenol Red Free DMEM F-12 supplemented with 5 % dextran treated FBS (DCC-FBS), 100 units/mL penicillin and 100 µg/mL streptomycin). The cells were incubated overnight at 37 $^{O}$C in a humidified atmosphere of 5 % $CO_2$. Successively, medium was removed and new assay medium was added. Test substances and positive controls were added using HP D300 Digital Dispenser (Tecan Group Ltd., Zürich, Switzerland). R1881 (Perkin Elmer, Skovlunde, Denmark) and hydroxyflutamide (OHF) (Toronto Research Chemicals, Toronto, Canada) was included in all independent experiments to ascertain assay performance in agonist and antagonist mode, respectively, in concentrations ranging from 0.002-2.7 nM and 31-8000 nM, respectively. In the antagonist mode of the assay, R1881 was added to all wells at a concentration of 0.1 nM. Test chemicals were tested in concentrations of 12.5, 25, 50, 100, and 200 µM. DMSO was used as vehicle control and was kept constant in all wells (0.2%) – a non-cytotoxic concentration (data not shown). The cells were incubated with test chemicals for 20-24 h.

Dual-Glo Luciferase Assay System from Promega Corporation (Madison, Wisconsin, USA) was used to measure firefly and renilla luciferase activity. Luminescence was measured on a LUMIstar® Galaxy luminometer (BMG LABTECH, Offenburg, Germany). 100 µL Dual-Glo® Luciferase Reagent was added to each well and the plates were placed on a horizontal shake for 10 min. The firefly luminescence was then measured. Successively, 60 µL/well of Dual-Glo® Stop & Glo® was added. After 10 minutes shaking luminescence was measured. Seven independent experiments were conducted for each test chemical and each exposure concentration was tested in triplicates within the independent experiment.

### 2.6.2 Aryl hydrocarbon receptor (AhR) reporter gene assay

The potential of the test substances to affect AhR activity was tested in an AhR reporter gene assay. The stably transfected rat hepatoma (H4IIE-CALUX) cells obtained from Dr. Michael Denison (University of California, USA) were used and the assay was performed as described previously (Rosenmai, Taxvig, Wedebye, Dybdahl, Vinggaard, Pedersen, et al., 2014).

Cells were cultured in Minimum Essential Medium alpha (MEMα) supplemented with 5% fetal bovine serum (FBS), 100 units/mL penicillin, 100 µg/mL streptomycin and 100 µg/mL fungizone. Medium components were supplied by InvitrogenTM, Life Technologies TM (Carlsbad, California, USA).

Cells were seeded in white clear-bottomed 96-well plates (Corning® Inc., Corning, New York, USA) at a concentration of 22 x $10^3$ cells/well in assay medium (MEMα supplemented with 1% FBS and 100 units/mL penicillin, 100 µg/mL streptomycin and 100 µg/mL fungizone). For cell viability studies, cells were seeded in black clear-bottomed 96-well plates (Corning® Inc., Corning, New York, USA) at a concentration of 11 x $10^3$ cells/well in assay medium. Cells were incubated for 24 h.

Successively, medium was exchanged and test substances and controls were added manually. Test substances were tested in nine 2-fold dilutions ranging from 0.8-200 µM. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) (AACN Standards) was used as a positive control and tested in concentrations ranging from 0.5-3000 pM. The vehicle was kept constant in all wells (0.2%) – a non-cytotoxic concentration (data not shown). The cells were incubated with test chemicals for 20-24 h.

At experiment termination, cells were lysed with 25 µL/well lysis buffer (25 mM of triphosphate (Sigma Aldrich), 15 % glycerol (VWR/BB), 1 % triton X (Sigma Aldrich), 1 mM dithiothreitol (Sigma Aldrich), and 8 mM $MgCl_2$ (Sigma Aldrich)) and left on shaker table for approximately 20 min. Successively, 40 µL/well luciferin solution were injected automatically and luminescence was measured on LUMIstar® Galaxy luminometer.

Cell viability was examined by use of resazurin. At experiment termination medium was removed and 100 µL of a 5 µg/mL resazurin solution (Sigma Aldrich) was added to each well. Plates were left to incubate for 3 h at 37 ºC, 5% $CO_2$, and a humidified atmosphere. Fluorescence was measured on EnSpire (Perkin Elmer) with an excitation and emission wavelength of 560 nm and 590 nm, respectively.

Three independent experiments were conducted for each test chemical with each exposure concentration in triplicates.

### 2.6.3 ANSA-TTR displacement assay

Binding of test chemicals to transthyretin (TTR) was examined in the ANSA-TTR displacement assay. The ANSA fluorophore (8-Anilino-1-naphthalene sulfonic acid ammonium salt) increases its fluorescence signal when bound to TTR, whereas the signal is reduced when ANSA is displaced by competition with thyroid hormones or exogenous substances.

Standard solutions in 1% DMSO were mixed in a black flat bottom 96-well plate (PerkinElmer, Skovlunde, Denmark) with 0.6 µM ANSA (Sigma Aldrich) and 0.5 µM TTR (Sigma Aldrich) in

PBS. Test substance concentrations were 50, 100 and 200 µM. After 2 h of incubation at 4°C, the plate was gently shaken for 10 s and fluorescence was measured (Enspire, Perkin Elmer). Negative controls only with 0.6 µM ANSA, ANSA-TTR positive controls, and T4 (thyroxine) (Sigma Aldrich) 0.156, 0.625 and 2.5 µM displacement controls were included on every plate. ANSA fluorescence was measured with excitation filter 380 ± 20 nm/emission filter 475 ± 20 nm). The experiment was repeated in three independent experiments with each exposure concentration tested in triplicates within each independent experiment.

#### 2.6.4 Data processing

For AR and AhR reporter assay data, each data point within the independent experiment was normalized to the mean of the plate controls. Successively, means from independent experiments were pooled. In the ANSA-TTR displacement assay, the fluorescence from the negative control was subtracted, and data were expressed as fluorescence relative to the ANSA-TTR maximal fluorescence (positive control). Each data point was normalized against the mean of the plate control and means from the three experiments were pooled.

Kruskal-Wallis test (Dunn's post hoc test) was used to examine differences between exposed groups and controls and a p-value of $<0.05$ was perceived as statistically significant. All data processing and statistical analyses were performed in GraphPad Prism 5 (GraphPad Software Ic, La Jolla, CA, USA).

## 3. Results and discussion

### 3.1 Migration assays by UPLC-QTOF

Cyclic esters were quantified by external calibration with AA-DEG and AA-DEG-IPA-DEG standards. The analytical parameters of UPLC-QTOF are shown in Table 2, including linearity, limit of detection (LOD) and limit of quantification (LOQ).

Table 1 summarizes the migration values (ng $g^{-1}$) of both cyclic esters in 20 different samples. The concentration of the cyclic esters in migration was highly variable but AA-DEG migration values were in all cases higher than the AA-DEG-IPA-DEG values. AA-DEG oligomer was in all migration samples between 20-994 ng $g^{-1}$ except for 17S that was below of limit of migration according to legislation (10 ng $g^{-1}$). However, AA-DEG-IPA-DEG oligomer was only present in concentration values between 4 and 346 ng $g^{-1}$ in 8 out of the 20 samples. To clarify, the detection

and quantification limits of the method were calculated and reported in Table 2 taking into account the dimension of the bags and the ratio $6dm^2$ per 1 kg simulant according to EU/10/2011.

For most multilayer materials, migration of the cyclic esters exceeded the migration limit established by EU/10/2011 (EC, 2011) for not-listed substances, which is 10 ng $g^{-1}$. Therefore, only the sample 17S should comply with the EU Regulation. Nevertheless, when a compound is not listed in the regulation, the Threshold of Toxicological Concern (TTC) approach can be used (EFSA, 2012). This approach assigns a theoretical toxicity class according to the compound chemical structure and Cramer rules (G. M. Cramer, Ford, & Hall, 1978). All the compounds are classified into three classes according to its toxicity: class I (low toxicity), class II (intermediate class) and class III (high toxicity), and a recommended value of maximum daily intake for each class is established (1.8, 0.54 and 0.09 mg/person/day, respectively). Toxtree software was used to estimate the theoretical toxicity of the cyclic esters. According to the TTC approach, both cyclic esters are classified as Cramer class III (high toxicity) and hence the maximum daily intake should be below 0.09 mg/person/day (G. M. Cramer, Ford, & Hall, 1978). The maximum recommended migration value according to the maximum daily intake can be calculated with the Estimated Daily Intake (EDI) equation described by FDA:

$$\text{EDI (mg/person/day)} = \text{Mig (mg·kg}^{-1}\text{)} \times 3\text{ kg} \times \text{CF} \qquad \text{Equation 1}$$

where 3 kg corresponds to the total food intake per person/day and CF is the consumption factor (daily fraction of food that is expected to be in contact with the packaging material). For adhesives, CF value is 0.14. Therefore, the maximum recommended migration for these compounds according to FDA would be **214 ng $g^{-1}$.**

According to EFSA (PlasticsEurope, 2014), the Estimated Daily Intake (EDI) equation is different:

$$\text{EDI (mg/person/day)} = \text{Mig (mg·kg}^{-1}\text{)} \times 1\text{ kg} \qquad \text{Equation 2}$$

where 1 kg corresponds to the total food eat per person/day. This equation is more restricted than the FDA equation. In this case, the maximum recommended migration for these compounds would be **90 ng $g^{-1}$**.

When using the TTC approach for risk assessment, the number of multilayer packaging materials that could be used is 6 out of 20, according to FDA, and 5 out of 20, according to EFSA.

In view of these results, gastric and intestinal digestions of the cyclic esters were performed. This study made it possible to obtain knowledge on the transformation processes of these compounds inside the human body and their bioaccessibilities.

### 3.2 Digestions assays

The aim of digestion assays was to examine if cyclic esters degraded in the stomach and intestine, thus decreasing their concentration and therefore reducing the amount of cyclic esters available to be absorbed by the body. Samples resulting from the digestion assays were analysed by UPLC-QqQ (MRM). Analytical parameters of UPLC-QqQ (MRM mode) of AA-DEG and AA-DEG-IPA-DEG standards are shown in Table 2.

The results showed that digestion led to a decrease in concentration of the cyclic polyesters. Figure 1 shows the percentage values of AA-DEG and AA-DEG-IPA-DEG oligomers after gastric (1a) and intestinal (1b) digestions at different time points ($t_0$, $t_{1h}$, $t_{2h}$ and $t_{4h}$). These data were normalized to a control with no digestion.

The amount of both cyclic oligomers progressively decreased during digestion. For AA-DEG, the final percentages of decrease were 31.2% (± 3.9) and 18.2 % (±3.5) after gastric and intestinal digestion, respectively. Gastric digestion was more effective than intestinal digestion. An overall summary of the AA-DEG digestion can be carried out taking into account that gastric digestion occurs first and intestinal digestion happens consecutively. The digestion resulted in an overall decrease of the parent compound of 43.7% (RSD<5%). On the other hand, for AA-DEG-IPA-DEG the final decrease after each digestion was higher, reaching 53.2 % (± 2.1) for gastric and 91% (± 6.8) for intestinal digestion, with an overall decrease of 95.8 % (RSD < 5%).

Digestion extracts were also analysed by UPLC-QTOF. Chromatograms showed the decrease of the oligomers peaks and, in addition, the emergence of new peaks with signals increasing with digestion. Figure 2 shows a chromatogram of a solution of AA-DEG (a) and AA-DEG-IPA-DEG (b) before ($t_0$) and after ($t_{4h}$) being submitted to a gastric digestion. A new peak was observed after digestion of AA-DEG , 5.31_257.099 (retention time_mass); and two peaks were observed after digestion of AA-DEG-IPA-DEG, 6.50_493.167 and 6.09_365.120. In intestinal digestion, the same analysis was carried out and the same new peaks were observed. When samples were analysed in negative mode, no differences between chromatograms before and after the digestion were observed.

According to their mass, 5.31_257.099 and 6.50_493.167 corresponded to the cyclic esters plus a water molecule. Its formation was the consequence of the hydrolysis of the cyclic esters and the opening of the ring due to the interaction with the gastric and intestinal simulants. This hypothesis is in agreement with previous studies (Gómez Ramos, Lozano, & Fernández-Alba, 2019; Úbeda, et al., 2017). Hydrolysed molecules always eluted before the parent molecule, as other authors have stated before (Úbeda, et al., 2017). AA-DEG high energy mass spectrum has been published in our own previous studies (Úbeda, et al., 2017). Figure 3 shows high collision energy mass spectra of AA-DEG-IPA-DEG (a) and its hydrolysed form (b) with their fragments. The spectra allowed the detection of the fragments and therefore its structure elucidation. Their common masses between cyclic and linear compound were 281.1040 and 193.0503 m/z.

The concentration of hydrolysed molecules in digestion assays was calculated using the cyclic oligomers as standards. Its evolution over time is shown in Figures 1c and 1d. Figure 1c shows concentration values of AA-DEG + H2O and AA-DEG-IPA-DEG + H2O during gastric digestion and Figure 1d shows concentration values of hydrolysed molecules during intestinal digestion. In gastric digestion, AA-DEG + H2O concentration increased to 86.7 ng $g^{-1}$ and AA-DEG-IPA-DEG + H2O to 175.4 ng $g^{-1}$. However, after intestinal digestion, AA-DEG + H2O concentration was below 6 ng $g^{-1}$ (LOD) and AA-DEG-IPA-DEG + H2O concentration was to 162.2 ng $g^{-1}$.

The compound 6.09_365.120, present in the digestion of AA-DEG-IPA-DEG, was identified as DEG-IPA-DEG, coming from a breakdown of an ester linkage of the cyclic oligomer. Its structure elucidation is shown in figure 3c.

It is important to highlight that the new compounds formed had lower toxicity according to Cramer rules (class I) which is a positive message. Transformations of cyclic esters to their opened form decreased their theoretical toxicity in most cases. Lower toxicity means a higher recommended daily intake (1.8 mg/person/day) and therefore, higher maximum recommended migration values, 4286 and 1800 ng $g^{-1}$ according to FDA and EFSA, respectively. According to the migration values in Table 1, all linear oligomers were below these limits and therefore no health risk for consumers would be expected.

On the other hand, the monomers (AA, DEG and IPA) were checked. The results showed that none of the monomers were present after the oligomer digestion assays above the limits of detection (LOD DEG= 3 ng $g^{-1}$, LOD AA=13 ng $g^{-1}$ and LOD IPA=5 ng $g^{-1}$).

Other compounds could have been formed due to the breakdown of the different ester linkages of the oligomers during the digestion process but they were below their detection limit.

### 3.3 In vitro endocrine assays

In the present study, AA-DEG-IPA-DEG showed a statistically significant antagonistic activity on AR at high concentrations (100 and 200 μM) with a maximum efficacy of approximately 25% decrease compared to vehicle control. AA-DEG led to a statistically significant antagonistic effect at 200 μM, however the maximum efficacy was approximately 10% compared to vehicle control (Figure 4). These effects occurred at non-cytotoxic concentrations. Comparatively, AA-DEG-IPA-DEG thus has greater antiandrogenic potential than AA-DEG. Neither of the test compounds exhibited any major effects in the AhR reporter gene assay (Supplementary material 1) nor the ANSA-TTR assay (Supplementary material 2).

To our knowledge, this is the first time AA-DEG-IPA-DEG and AA-DEG have been tested for ability to interfere with AR, AhR, and TTR. However, the monomers DEG and IPA have been tested for AR binding both in silico and in vitro, as well as in an AR transactivation assay, but exhibited no effect (Osimitz, Welsh, Ai, & Toole, 2015). These findings could suggest that the AA moiety of the compounds play a role in the observed antiandrogenic activities.

As a next step, we preliminarily evaluated whether the metabolites of the cyclic esters exhibited any AR antagonism. The results indicated that no active metabolites were formed at concentrations up to 12.5 μM of parent compound, suggesting that the parent compounds were responsible for the activity (data not shown).

The concentrations leading to antiandrogenic activity (AA-DEG: 200 μM; AA-DEG-IPA-DEG: 100-200 μM) are greater than the migration values of the compounds under the assumption of 1 kg food intake per day containing the highest migration distributed in 5 L blood (higher migration value of AA-DEG: 994 ng $g^{-1}$=> 0.92 μM; and of AA-DEG-IPA-DEG: 346 ng $g^{-1}$=> 0.15 μM). This suggests that the migration from a single FCM to food would not lead to a concentration that could cause inhibition of AR activity. However, humans may be exposed to oligomers from multiple FCMs simultaneously, as well as other sources, thereby increasing the exposure to these substances. In addition, multiple substances have been reported antiandrogens (Vinggaard, Niemelä, Wedebye, & Jensen, 2008), which can exert mixture effects when exposure occur simultaneously (Metzdorff, Dalgaard, Christiansen, Axelstad, Hass, Kiersgaard, et al., 2007; Orton, Ermler, Kugathas, Rosivatz, Scholze, & Kortenkamp, 2014). Therefore, a better understanding of human exposure sources as well as human levels are needed in future studies.

## 4. Conclusions

The migration values of the cyclic polyesters that are formed during PU manufacturing (AA-DEG and AA-DEG-IPA-DEG), was highly variable for the different multilayer materials studied. The PU manufacturing process together with the physico-chemical materials properties and the migration conditions could be the explanation for these differences. Besides, results showed that AA-DEG migrated more than AA-DEG-IPA-DEG, probably due to its smaller structure and the absence of the aromatic ring.

The digestion studies showed that the cyclic esters were degraded significantly after gastric and intestinal digestion, which was very positive because their bioaccessibility to the human body became lower. In addition, the new compounds formed had lower toxicity according to Cramer rules, what was also positive from a food safety and human health perspective.

The digestion processes affected the two cyclic esters differently. In the case of AA-DEG, gastric digestion influenced the most with a decrease of 31%, whereas in the case of AA-DEG-IPA-DEG, the influence of intestinal digestion was greater (decrease of 91%). Global digestion (gastric plus intestinal digestion) was more dominant for AA-DEG-IPA-DEG than for AA-DEG. This means that the bioaccessibility of AA-DEG-IPA-DEG is expected to be lower than of AA-DEG.

Regarding to the endocrine activity, slight effects were observed on AR activity at higher test concentrations suggesting that the compounds can act as AR antagonists. When comparing the compounds, AA-DEG had lower antagonistic activity than AA-DEG-IPA-DEG. This can be hypothesized to be due to the fact that this last compound has a phthalate as part of its chemical structure. Monomers have so far shown no toxicity but their oligomers has slightly AR activity. No effect on TTR binding or AhR activity was found. It may be hypothesized that this lack of effects in vitro might be due to the large size of these molecules that may hinder accessibility to the target.

It would be interesting to perform a broader in vitro screening to expand the toxicological knowledge on these compounds.

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Potential conflicts of interest do not exist

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## Figure captions

**Fig 1.** Decrease percentage evolution of AA-DEG and AA-DEG-IPA-DEG oligomers for gastric (a) and intestinal (b) digestion over time ($t_0$, $t_{1h}$, $t_{2h}$ and $t_{4h}$). Evolution of concentration of AA-DEG + $H_2O$ and AA-DEG-IPA-DEG + $H_2O$ oligomers for gastric (c) and intestinal (d) digestion over time ($t_0$, $t_{1h}$, $t_{2h}$ and $t_{4h}$).

**Fig 2.** Chromatograms of AA-DEG (a) and AA-DEG-IPA-DEG (b) in gastric digestion assays at time 0 and after 4 hours by UPLC- QTOF.

**Fig 3.** High collision energy spectra for AA-DEG-IPA-DEG (a), its hydrolysed form (b) and a fragmentation product, DEG-IPA-DEG (c)

**Fig 4.** Agonism, antagonism and cytotoxicity data from the androgen receptor reporter gene assay of AA-DEG-IPA-DEG (up) and AA-DEG (down) oligomer. Data presented normalized to the vehicle control as pooled means from 7 independent experiments (mean ± SD, n=7). *indicates significant differences ($p < 0.05$).

Table 1: Migration values (ng $g^{-1}$) of AA-DEG and AA-DEG-IPA-DEG in different food simulants from different materials with polyurethane adhesive.

| Material code | Structure | Uses | Migration conditions / Simulant | Concentration AA-DEG (ng $g^{-1}$ simulant) | Concentration AA-DEG-PA-DEG (ng $g^{-1}$ simulant) |
|---|---|---|---|---|---|
| **1S** | PET/PE | BF | 3 days at 40 ºC / Water | 529 ± 67 | <1.0 |
| **2S** | PET/PE | BF | 3 days at 40 ºC / Water | 496 ± 40 | <1.0 |
| **3S** | PET/PE | BF | 3 days at 40 ºC / Water | 275 ± 170 | <1.0 |
| **4S** | PET/PE | BF | 3 days at 40 ºC / Water | 698 ± 4 | 56 ± 10 |
| **5S** | PET/PE | BF | 3 days at 40 ºC / Water | 566 ± 55 | 4.0 ± 0 |
| **6S** | PET/PE | BF | 3 days at 40 ºC / Water | 428 ± 70 | <3.0 |
| **7S** | PET/PE | BF | 3 days at 40 ºC / Water | 994 ± 201 | 346 ± 59 |
| **8S** | PET/PE | BF | 3 days at 40 ºC / Water | 360 ± 2 | <1.0 |
| **9S** | PET/PE | BF | 3 days at 40 ºC / Water | 530 ± 10 | <1.0 |
| **10S** | PET/PE | BF | 3 days at 40 ºC / Water | 350 ± 20 | <1.0 |
| **11S** | Unknown | FC | 10 days at 60ºC / 10% ethanol | 20 ± 5 | <1.0 |
| **12S** | cPP/Al | FC-pasteurization | 30 minutes at 121 ºC / 95% ethanol | 72 ± 12 | <1.0 |
| **13S** | Unknown | FC-pasteurization | 30 minutes at 121 ºC / 95% ethanol | 30 ± 2 | <1.0 |
| **14S** | PET/Alu/OPA/cPP | FC-pasteurization | 30 minutes at 121 ºC / 95% ethanol | 342 ± 10 | 98 ± 8 |
| **15S** | PET/Alu/PE | FC | 10 days at 60 ºC / 95% ethanol | 93 ± 6 | 14 ± 4 |
| **16S** | Alu/PE | FC | 10 days at 60ºC / 95% ethanol | 51 ± 12 | <1.0 |
| **17S** | OPA/cPP | FC-pasteurization | 30 minutes at 121 ºC / 95% ethanol | <1.5 | <1.0 |
| **18S** | PET/Alu/PE | FC-pasteurization | 30 minutes at 121 ºC / 95% ethanol | 443 ± 89 | 261 ± 30 |
| **19S** | Unknown | FC-pasteurization | 30 minutes at 121 ºC / 95% ethanol | 535 ± 16 | 250 ± 60 |
| **20S** | PET/Al/PA/CPP | FC | 10 days at 60 ºC / 95% ethanol | 759 ± 72 | 12 ± 2.0 |

BF: biological fluids; FC: food contact. Al: aluminum; CPP: cast polypropylene; PET: polyethylene terephthalate; OPA: oriented polyamide; PE: polypropylene; PA: polyamide.

Table 2: Analytical parameters of UPLC-QTOF and UPLC-QqQ (MRM) analysis

| Parameters | UPLC-QTOF | | UPLC-QqQ (MRM) | |
|---|---|---|---|---|
| | AA-DEG | AA-DEG-IPA-DEG | AA-DEG | AA-DEG-IPA-DEG |
| Linear range (ng $g^{-1}$) | 15-1240 | 10-1320 | 17.7-1135 | 1.1-1071 |
| $R^2$ | 0.9994 | 0.9980 | 0.9990 | 0.9999 |
| LOD (ng $g^{-1}$) | 5 | 3.3 | 5.9 | 0.4 |
| LOQ (ng $g^{-1}$) | 15 | 10 | 17.7 | 1.1 |

AA: adipic acid, DEG: diethylene glycol, IPA: isophthalic acid

**Fig 1.** Decrease percentage evolution of AA-DEG and AA-DEG-IPA-DEG oligomers for gastric (a) and intestinal (b) digestion over time ($t_0$, $t_{1h}$, $t_{2h}$ and $t_{4h}$). Evolution of concentration of AA-DEG + H2O and AA-IDEG-PA-DEG + H2O oligomers for gastric (c) and intestinal (d) digestion over time ($t_0$, $t_{1h}$, $t_{2h}$ and $t_{4h}$).

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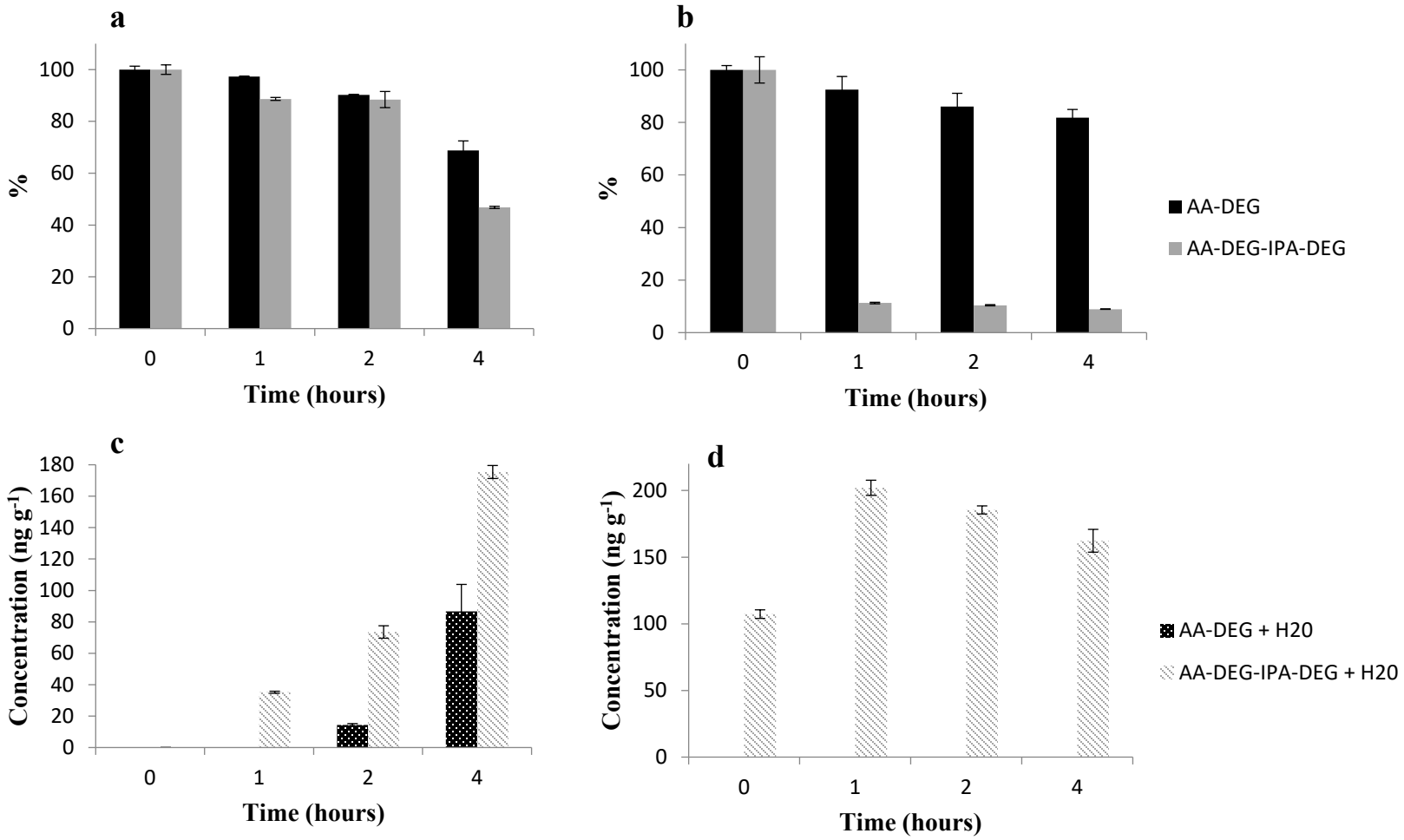
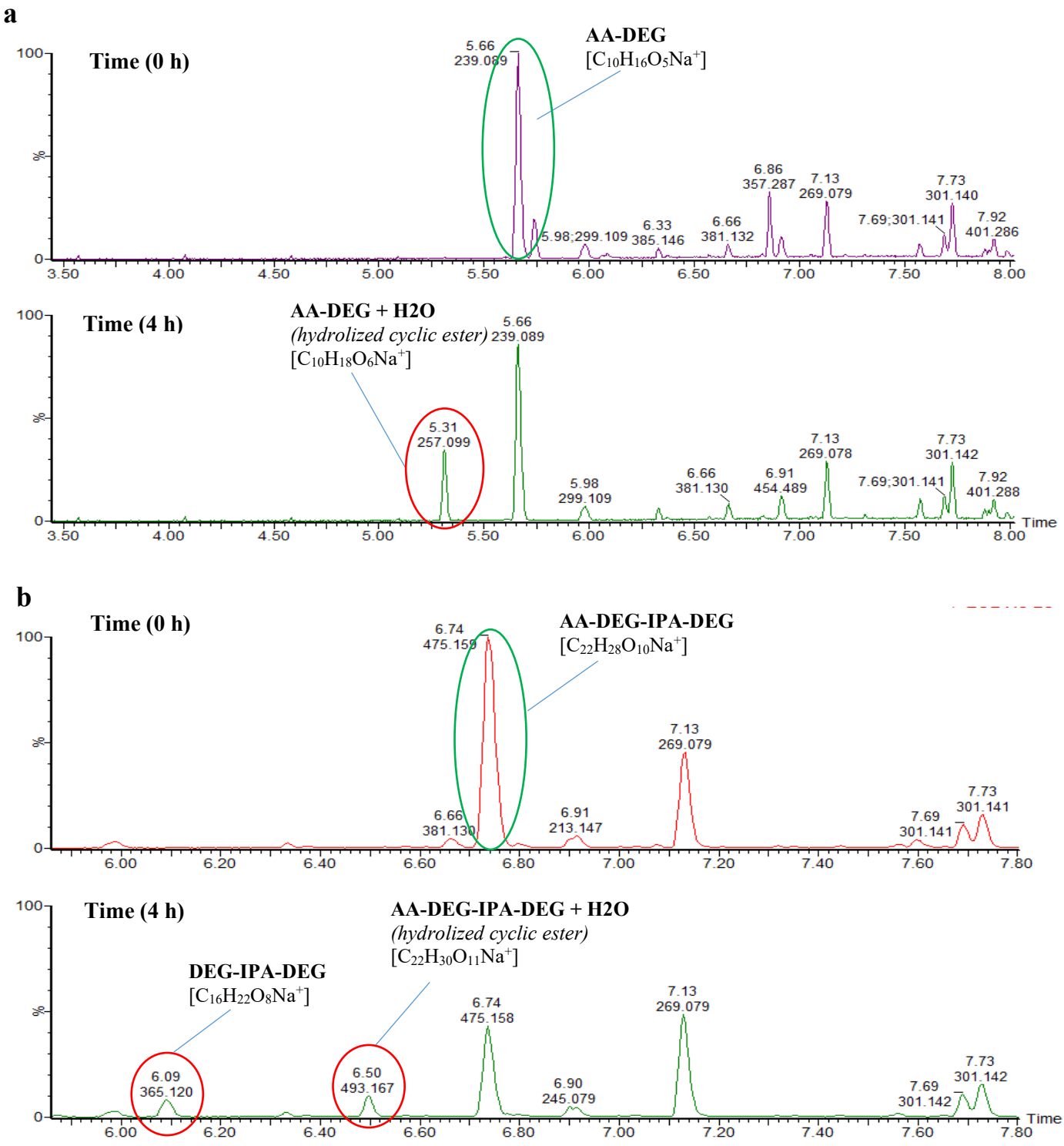
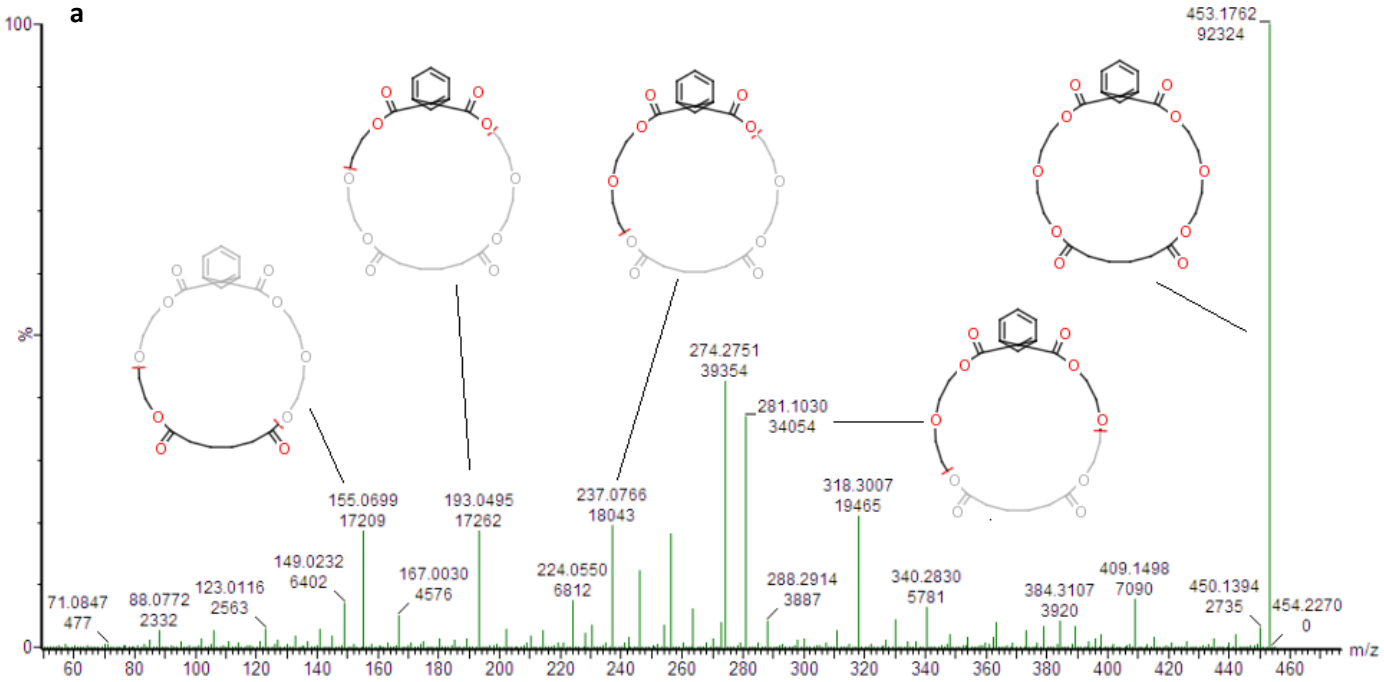
**Fig 3.** High collision energy spectra for AA-DEG-IPA-DEG (a) and its hydrolyzed form (b).

**Fig 4.** Agonism, antagonism and cytotoxicity data from the androgen receptor reporter gene assay of AA-DEG-IPA-DEG (up) and AA-DEG (down) oligomer. Data presented normalized to the vehicle control as pooled means from 7 independent experiments (mean ± SD, n=7). *indicates significant differences ($p < 0.05$).


a
b
c
d
%
Concentration (ng g-1)
Time (hours)
0
1
2
4
AA-DEG
AA-DEG-IPA-DEG
AA-DEG + H20
AA-DEG-IPA-DEG + H20


a
Time (0 h)
AA-DEG
[C10H16O5Na+]
5.66
239.089
5.98;299.109
6.33
385.146
6.66
381.132
6.86
357.287
7.13
269.079
7.69;301.141
7.73
301.140
7.92
401.286
Time (4 h)
AA-DEG + H2O
(hydrolized cyclic ester)
[C10H18O6Na+]
5.66
239.089
5.31
257.099
5.98
299.109
6.66
381.130
6.91
454.489
7.13
269.078
7.69;301.141
7.73
301.142
7.92
401.288
Time
b
Time (0 h)
AA-DEG-IPA-DEG
[C22H28O10Na+]
6.74
475.159
6.66
381.130
6.91
213.147
7.13
269.079
7.69
301.141
7.73
301.141
Time (4 h)
AA-DEG-IPA-DEG + H2O
(hydrolized cyclic ester)
[C22H30O11Na+]
DEG-IPA-DEG
[C16H22O8Na+]
6.09
365.120
6.50
493.167
6.74
475.158
6.90
245.079
7.13
269.079
7.69
301.142
7.73
301.142
Time


a
100
%
0
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92324
274.2751
39354
281.1030
34054
318.3007
19465
155.0699
17209
193.0495
17262
237.0766
18043
149.0232
6402
167.0030
4576
224.0550
6812
288.2914
3887
340.2830
5781
384.3107
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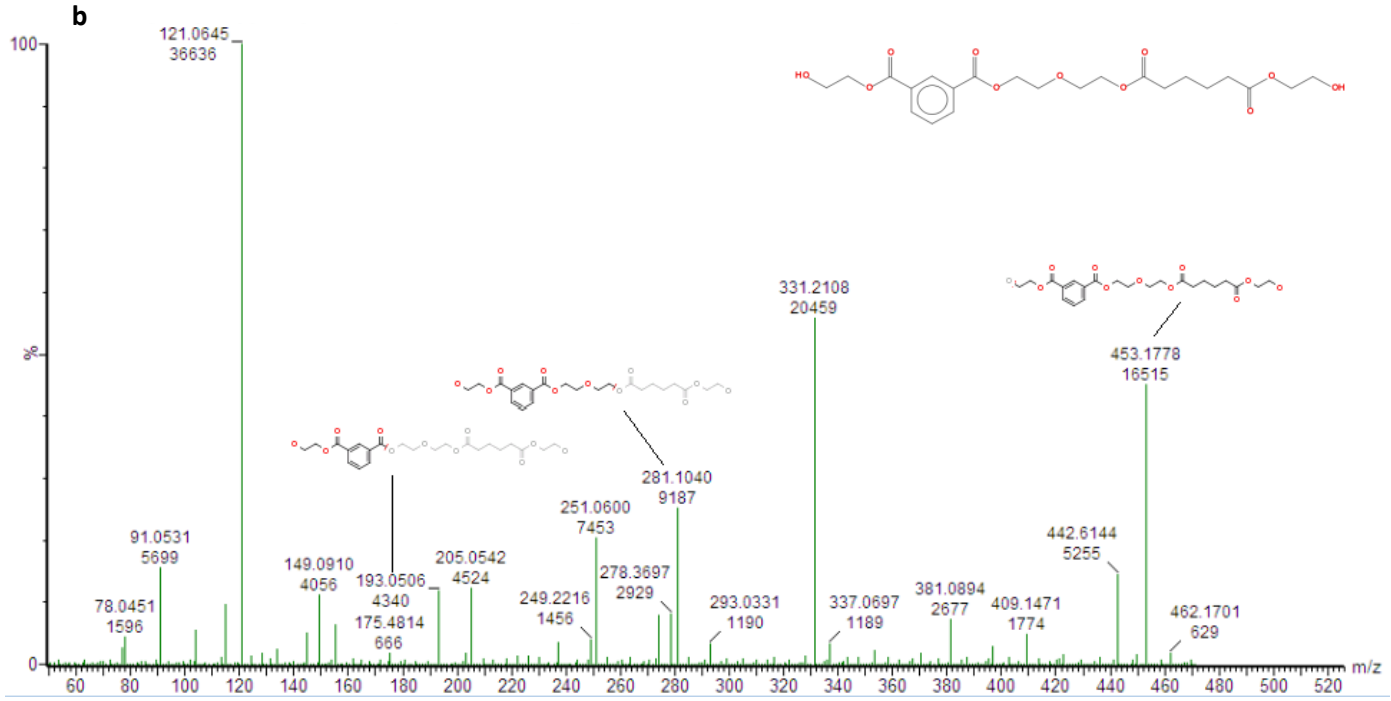
409.1498
7090
450.1394
2735
454.2270
0
123.0116
2563
88.0772
2332
71.0847
477
60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 400 420 440 460
m/z


b
100
%
0
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36636
331.2108
20459
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16515
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7453
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5699
149.0910
4056
205.0542
4524
193.0506
4340
278.3697
2929
249.2216
1456
293.0331
1190
337.0697
1189
381.0894
2677
409.1471
1774
442.6144
5255
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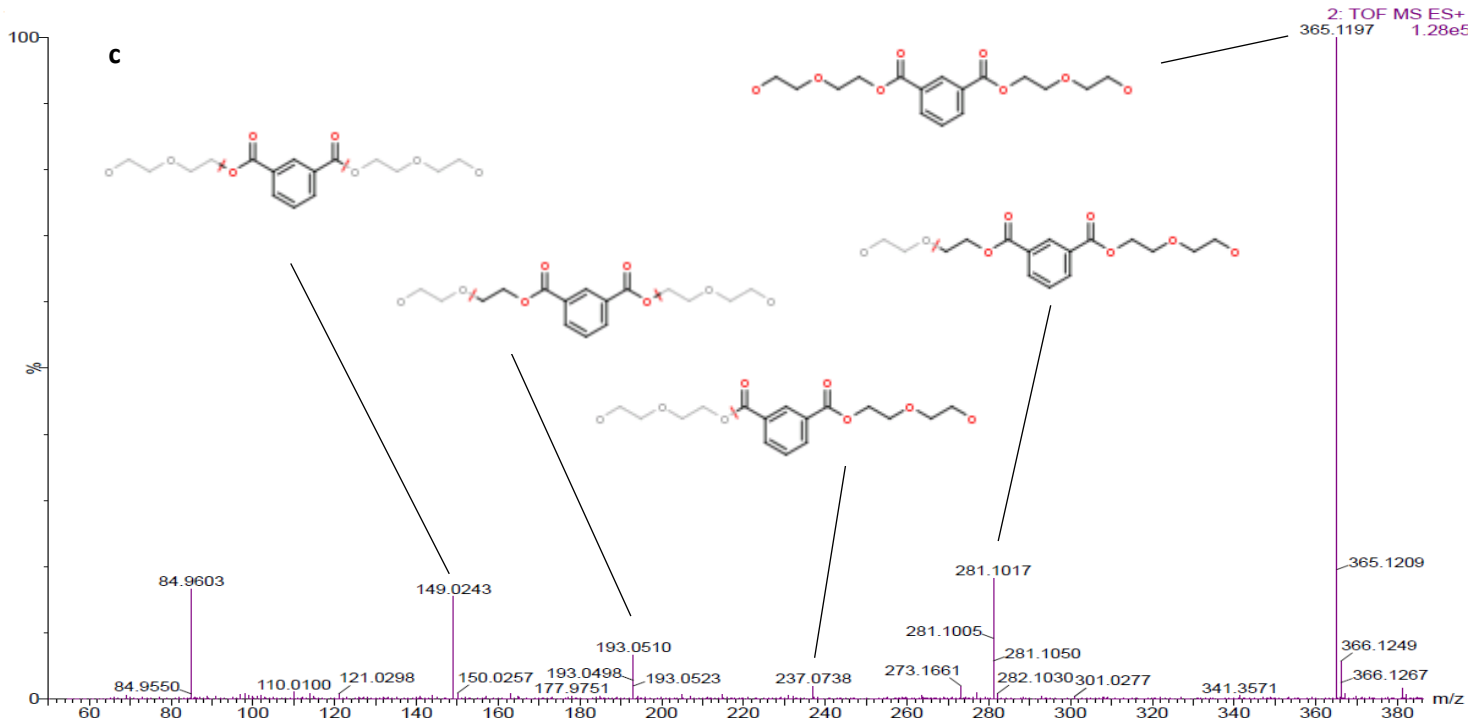
666
60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 400 420 440 460 480 500 520
m/z


c
2: TOF MS ES+
365.1197
1.28e5
100
%
0
84.9603
149.0243
193.0510
281.1017
365.1209
281.1005
281.1050
366.1249
366.1267
84.9550
110.0100
121.0298
150.0257
193.0498
177.9751
193.0523
237.0738
273.1661
282.1030
301.0277
341.3571
m/z
60
80
100
120
140
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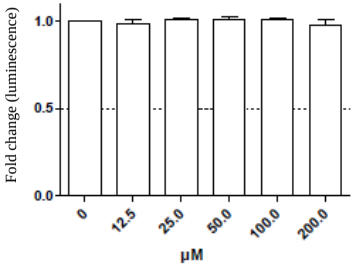
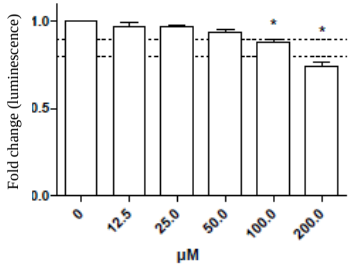
180
200
220
240
260
280
300
320
340
360
380

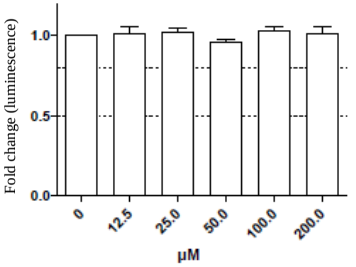
Agonism AA-DEG-IPA-DEG



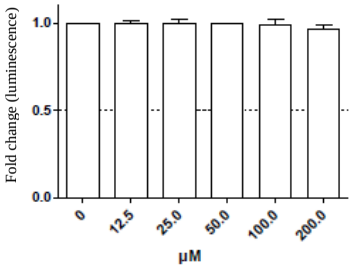

Antagonism AA-DEG-IPA-DEG



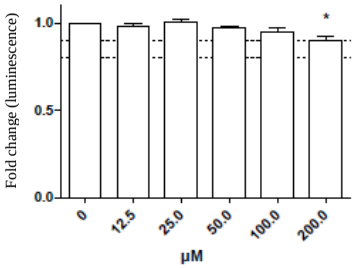

Cytotoxicity AA-DEG-IPA-DEG



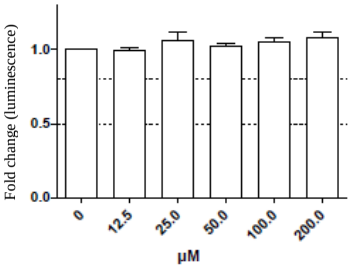

Agonism AA-DEG



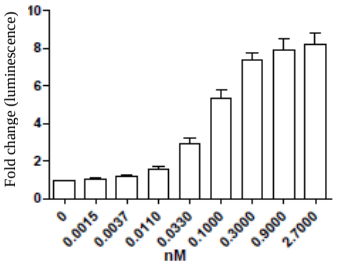

Antagonism AA-DEG



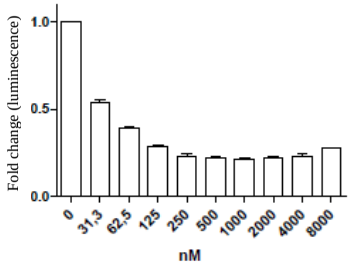

Cytotoxicity AA-DEG




R1881




OHF




Marking of * represents a statistically significant level of p<0,05.