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Synthesis and quantification of oligoesters migrating from starch-based food packaging materials

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Abbreviations: UPLC-MS, ultra-performance liquid chromatography-mass spectrometry; TTC, threshold of toxicological concern; NIAS, Non-intentionally added substances; IAS, Intentionally added substances; PLA, poly(lactic acid) ; PVA, polyvinyl alcohol; PBAT, poly(butylene adipate-co-terephthalate).

Abstract

The term oligomer refers to structurally diverse compounds coming from incomplete polymerisation or polymer degradation. Their ability to migrate into foodstuffs along with recent studies about their bioavailability and toxicity have risen concerns about the scarcity of standards necessary to perform analytical and toxicological studies. In this work, migration extracts of three starch-based biopolymers films destined to be in contact with fruits and vegetables were analysed according to European legislation 10/2011. UPLC-MS(QTOF) analysis allowed to identify a majority of oligoesters as non-intentionally added substances. A stepwise synthesis approach was used to synthesise and isolate eleven cyclic and linear oligoester standards ranging from 2 to 8 monomers based on adipic acid, 1,4-Butanediol, isophthalic acid and propylene glycol monomers. Characterisation was performed by ^1H and ^{13}C NMR as well as high resolution mass spectrometry. An overall high purity of > 98 % was achieved as detected by UPLC-MS(Orbitrap). The standards were then used to unequivocally identify the oligoesters in the samples by comparing their UPLC-MS/MS spectra and to quantify or semi-quantify the migrant oligoesters in the samples. The results deemed safe only one out of the three biopolymer films according to the threshold of toxicological concern concept. The work herein described aims to contribute towards the oligomers knowledge gaps, opening the door for comprehensive risk and absorption, distribution, metabolism, excretion and toxicity (ADMET) assessments.

Keywords: NIAS, oligoesters, biopolymers, migration, food packaging

Highlights

- Non-targeted LC-HRMS investigation migrants from FCM starch-based biopolymers.
- Oligoesters appeared as the dominant NIAS.
- Oligomers from AA, BD, iPA and PG were identified in the samples.
- Stepwise synthesis of eleven cyclic and linear oligomers standards composed of 2 to 8 monomers .
- Using oligoester standards to quantify, only one biopolymer sample was compliant with legislation.
- Quantification raises concern in 2 out of the 3 samples according to the TTC concept.

1. Introduction

Regarding plastics, the prefix bio- can refer to polymers coming from renewable sources or having a biological origin, their biodegradability or compostability, or a combination of both (Rosenboom et al., 2022). This feature, together with acceptable physical and mechanical properties and a low carbon footprint, contributes to their increasing market growth rate (Lampinen, 2010). Whilst plastic applications comprise consumer goods, electronics, agriculture, etc., the packaging sector represents the major market (48%) for bioplastic materials (Romero García et al., 2022).

Starch-based biopolymers are one of the most popular (about 18% of market share) bio-based plastic choices (Romero García et al., 2022). Being widely available, starch has to be mixed with plasticizers and other chemical moieties to improve their physical properties. The incorporation of reinforcements, chemical modifications and the blending with other copolymers is a common practice to minimize the handicaps of starch-based materials such as poor mechanical properties or high hydrophilicity (Agarwal et al., 2023). When it comes to food packaging applications, research has focused on developing starch-based blends containing poly(lactic acid) (PLA), polyvinyl alcohol (PVA) and poly(butylene adipate-co-terephthalate) (PBAT) among others, to improve both their physical and chemical properties (García-Guzmán et al., 2022). However, the presence of a large and diverse number of blends represents a challenge for the risk assessment of both intentionally added substances (IAS) and non-intentionally added substances (NIAS).

IAS as well as NIAS can be transferred from a packaging material into the food, making migration tests a widely applied tool for the proper risk assessment of a plastic food contact material (EU10/2011). IAS are usually well under control with fulfilled risk assessments and defined specific migration limits (Groh et al., 2021). However, as diverse substances coming from impurities in the raw materials, incomplete polymerization, or polymer degradation, NIAS are still being discovered, especially in new food packaging materials (Aznar et al., 2019; E.L. Bradley, 2010; Hayrapetyan et al., 2024; Ubeda et al., 2021; Vázquez-Loureiro et al., 2023). Oligomers, low molecular weight polymers, constitute one of the primary forms of NIAS (Shi et al., 2023). Due to their low molecular weight (generally below 1,000 Da), they can migrate from the material matrix into the food and are often overlooked by polymer

scientists, who focus their attention on the 10^4 up to 10^6 Da range (Shi et al., 2023). As most biopolymers are formed by a polycondensation reaction of various monomers, structurally and chemically diverse oligoesters often represent the dominant form of NIAS (Ubeda et al., 2021).

Lack of isolated oligoester standards results in an analytical challenge for the identification and quantification of NIAS (Nerin et al., 2013; Omer et al., 2018). Moreover, it limits the capability to perform risks assessments that would shed the light on the human and environmental exposure and absorption, distribution, metabolism, excretion and toxicity (ADMET) of the oligoesters. Hence, in recent years, efforts have been made to contribute towards the availability of migrant oligoesters (Cariou et al., 2022; Paseiro-Cerrato et al., 2016; Pietropaolo et al., 2018). Nevertheless, the need for oligoester reference standards is nothing but increasing.

In the present study, migration extracts of three starch-based biopolymer films destined to be in contact with fruits and vegetables were analysed by non-targeted LC/HRMS. From this, a variety of eleven linear and cyclic oligoester combinations was identified as possible NIAS. composed by 1,4-butanediol, propylene glycol, phthalic acid and adipic acid. Based on a stepwise synthetic strategy, they were synthesized, and they were used to unequivocally confirm and quantify the NIAS oligoesters migrating from the biopolymer samples.

2. Materials and methods

2.1. Reagents

Reagents used for analytical and migration purposes as well as for synthetic purposes are provided in the supplementary information.

2.2. Samples

The food safety of three commercial starch-based films (S1, S2 and S3) for food packaging applications were investigated in this study. Samples were provided by a local manufacturer and their formulation was not disclosed. Their thicknesses were measured using a digimatic micrometer from Mitutoyo (Kanagawa, Japan) as being 26.5 ± 1.1 , 25.5 ± 1.8 and 29.8 ± 2.4 μm , respectively.

2.3. Migration tests

All the migration experiments were performed in triplicate and according to the European Regulation for food contact materials EU/10/2011 (European Commission, 2011). Migration tests were performed using three different food simulants: ethanol 10% (v/v, simulant A), acetic acid 3% (w/v, simulant B) and Tenax[®] (simulant E). For simulants A and B, migration tests were performed by total immersion of cut-offs of 5 × 1 cm in 20 mL vials which were filled according to the 6 dm² contact surface/kg of simulant rate, established by the Regulation EU/10/2011. For simulant E migration experiments, cut-offs of 4 × 2 cm were placed in direct contact with 0.32 grams of Tenax[®] inside aluminium foil pouches following the 4 g.dm⁻² ratio established by UNE-EN-14338 (AENOR, 2004) and placed inside glass Petri dishes. Migration experiments took place in an oven at 40 °C during 10 days. Migration extracts from simulants A and B were directly injected in the UPLC-MS(QTOF) system. Prior to injection, Tenax[®] from each migration experiment was extracted twice with ethanol following the methodology designed by Vera et al. (Vera et al., 2011). The recovered ethanol was then filtered with a PTFE syringe filter (0.45 µm) and concentrated to approximately 0.5 mL under a gentle stream of nitrogen. Concentration step was gravimetrically monitored.

2.4. UPLC-HRMS analysis of migration extracts

Chromatographic separation was carried out on a CORTECS UPLC BEH C18 column (1.6 µm, 2.1 × 100 mm) using an UPLC Acquity system, both from Waters (Milford, MA, USA). Chromatography was performed at 0.3 mL.min⁻¹ column flow using water (phase A) and methanol (phase B) both with 0.1% (v/v) formic acid as mobile phases. Column temperature was set at 35 °C and injection volume was 10 µL. Chromatography started at A/B 95:5 (v/v), changed to 5:95 in 6 min and stayed at these conditions for an additional 4 min, going back to the initial 95:5 conditions to pre-condition the column for 3 min.

The UPLC system was connected through an ESI probe to a Xevo G2 QTOF mass spectrometer from Waters. Instrument configuration was as follows: capillary at 2.8 kV, sampling cone at 35 V, extraction cone at 3 V, source temperature at 150 °C, desolvation temperature at 450 °C, cone gas flow at 40 L h⁻¹ and desolvation flow at 600 L h⁻¹. Acquisition was carried out in

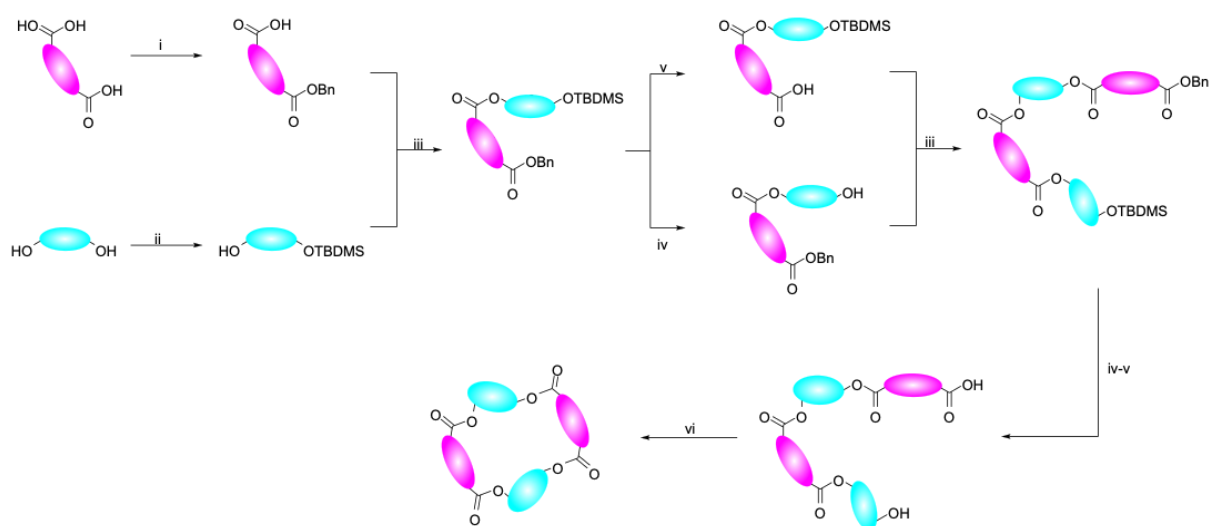
sensitivity MS^E mode, allowing the acquisition over a range of collision cell energies (CE) from 15 to 30 V during the same run. Data were recorded using Masslynx[®] v4.1 software.

The identification of compounds was performed by comparing migration extracts with a migration blank and following a previously described methodology (Aznar et al., 2016) to achieve level 2b of the scale proposed by Schymanski et al. (Schymanski et al., 2014). Level 5 (lowest) comprises an accurate measurement of the mass-to-charge ratio (m/z). Level 4 is achieved with an unambiguous molecular formula. Level 3 is obtained when multiple potential structures are feasible. Level 2 can be claimed by the proposal of a single structure supported by experimental diagnostic evidence such as MS² (2b) or matching library spectra (2a). Final confirmation of the structure (level 1) is only reached through comparison with a reference standard. Briefly, using the low energy spectrum, the precise mass of precursor ions was used to determine the lowest mass error and the highest isotopic fit of the elemental composition options proposed by Masslynx. Afterwards, the selected elemental compositions were linked to a chemical structure using different chemical databases (e.g. Chemspider, Scifinder) and freely available software (NIAS-db 1.0, Cariou et al., 2022) by paying attention to the chemical criteria and background experience about NIAS and IAS in bio-based polymers. Finally, a candidate molecule was selected using its high-energy mass spectrum. For being selected as a candidate, at least two main fragment ions of the high energy mass spectrum showed a score value below 3 using the MassFragment[®] tool from Masslynx. The score value was calculated by the software based on fragmentation probabilities.

2.5. Synthesis and characterization of oligoesters standards

The selection of oligoester candidates to be synthesized was made with regards to obtain as many structurally diverse oligoesters as possible but firstly considering the hypothesised oligoesters in the migration extracts. An optimized stepwise oligoester synthesis strategy previously described (Cariou et al., 2022) was readapted for the preparation of new identified substances arising from the above-mentioned migration protocols. . The synthesis of these oligoester standards involves the use of new diol monomers and new diacid counterparts. Scheme 1 briefly summarises all reactions in the stepwise synthesis sequentially implemented involving successive monosilylation, monobenylation, debenylation, desilylation, esterification and macrolactonization. Products were purified after each reaction by flash

column chromatography by an automatic Reveleris Büchi apparatus (Flawil, Switzerland) using pre-packed high purity 40 μm silica cartridges (4 to 220 grams, Büchi). An in-depth description of the synthesis protocol, monitoring, and characterisation equipment (^1H and ^{13}C NMR, ESI-TOF-HRMS) is provided in the supplementary information.



Scheme 1 – Reaction sequence involved in the stepwise synthesis of oligoester standards. Method A: (i) BnBr, NaHCO_3 , Dioxane/DMF ; Method B: (ii) TBDMSCl, Et_3N , DMAP, DCM ; Method C: (iii) EDC.HCl, DMAP, DCM ; Method D: (iv) HF.Pyr, THF ; Method E: (v) H_2 , $\text{Pd}(\text{OH})_2$, *i*PrOH ; Method F: (vi) 2,4,6-Trichlorobenzoyl chloride, Et_3N , DMAP, 10^{-3}M in THF.

2.6. Purity assessment of oligoesters standards

To perform a purity assessment of the synthesized oligoester compounds, each standard was first solubilised with DCM and further gravimetrically diluted with ACN until a $10\ \mu\text{g.g}^{-1}$ solution. Each solution was characterised on a UHPLC UltiMate 3000 coupled to an Orbitrap Q Exactive instrument fitted with a heated electrospray ionisation source (UHPLC-ESI-MS-Orbitrap), both from Thermo Scientific (Waltham, MA, USA). Chromatographic separation was achieved at $40\ ^\circ\text{C}$ on a C18 Hypersil Gold column ($1.9\ \mu\text{m}$, $2.1 \times 100\ \text{mm}$) from Thermo Fisher Scientific (San José, CA, USA). The flow rate was set at $0.4\ \text{mL.min}^{-1}$ and the mobile phase was composed of 10 mM ammonium acetate in both water (A) and acetonitrile (B). Separation began with A/B 95:5 (1 min) and ramped to 100:0 over 16 min, to be maintained over 9 minutes before going back to the initial conditions (2 min). The ionization parameters were as follows: sheath gas flow, 50 arbitrary units (AU); auxiliary gas flow, 10 AU; capillary temperature, $350\ ^\circ\text{C}$; heater temperature, $300\ ^\circ\text{C}$; spray voltage, 3.5 kV; S-lens radio frequency, 70 AU. Data were acquired in full scan by using the positive/negative switching

mode over the m/z range 100 – 1,064 at a nominal resolving power of 70,000. Automatic gain control (AGC Target) was set at high dynamic range (1×10^5) and maximum injection time (IT) at 250 ms. Purity percentage was determined by measuring the peak area of the $[M + H]^+$, $[M + Na]^+$, $[M + K]^+$, $[M + NH_4]^+$, $[M - H]^-$ and $[M + HAc - H]^-$ present adducts of the oligoester and the impurities.

2.7. Identification and quantification of oligoesters in migration extracts

In the present study, synthesized oligoesters standards were used to achieve Schymanski's level 1 identification, putting together the information obtained from section 2.4. and the relative retention time, chromatographic peak shape and MS^2 fragmentation pattern. To this end, migration extracts, migration blanks and a $10 \mu\text{g g}^{-1}$ solution containing the synthesized oligoesters were analysed by UPLC-MS(QTOF). Chromatographic parameters and instrument configuration were kept the same as in section 2.3. However, data acquisition was performed using the MS^2 function by selecting the most abundant adduct (as observed in full scan mode) as parent ion to then apply a CE potential ramp (20 to 50 V) in order to favour both low and high mass fragments. To avoid co-eluting interferences, only one parent ion was fragmented at each time window.

Quantification was conducted using the external calibration method. To account for impurities between oligoester standards, different sets of 14 points calibration curves were gravimetrically prepared at the following concentrations: 10,000, 5,000, 2,500, 1,000, 750, 500, 300, 150, 100, 50, 20, 10, 5 and 1 ng.g^{-1} . The analysis method was the same employed for the analysis of migration samples in section 2.3. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated as the smallest concentration of analyte that provided a signal to noise ratio three times and ten times the blank respectively.

3. Results and discussion

3.1. NIAS migration in starch-based biopolymer films

Detected compounds in food simulants A, B and E after contact with the three starch-based biopolymer film samples are shown in Table 1. No IAS were detected in any of the samples through the non-targeted analysis. However, 22 oligoester combinations were identified as suggested by MassFragment, which accounted for 90% of the cumulative area of signals after

blank subtraction ($n = 23, 175 \times 10^3$ AU). Migrant oligoesters added up to 97%, 96% and 85% in simulant A ($n = 11, 75 \times 10^3$ AU), B ($n = 18, 54 \times 10^3$ AU) and E ($n = 10, 46 \times 10^3$ AU) respectively.

In terms of units, even cyclic combinations were dominated by 4-unit combinations ($n=4, 91 \times 10^3$ AU), followed by 6-units ($n=2, 13 \times 10^3$ AU), 8-units ($n=2, 9 \times 10^3$ AU) and 2-units ($n=2, 8 \times 10^3$ AU). The only odd cyclic combinations hypothesised were composed of lactic acid monomers only and were a 7-units (11×10^3 AU) and a 5-units (3×10^3 AU). Linear oligoesters were less abundant, ranging from 2 to 7 units, the most intense being the 5-units ($n=2, 13 \times 10^3$ AU), 2-units ($n=2, 10 \times 10^3$ AU) and 4-units ($n=2, 8 \times 10^3$ AU). Only one combination for each of the 3-units (2×10^3 AU), 6-units (0.1×10^3 AU) and 7-units (0.2×10^3 AU) could be found. It could be withdrawn that cyclic oligoesters are favoured compared to linear oligoesters being diols or hydroxy acids. No diacid linear combinations were found, maybe due to their properties and to their higher reactivity. Oligoester combinations involved 5 diols: butanediol (BD), ethylene glycol (EG), propylene glycol (PG), diethylene glycol (DEG) and trimethylolpropane (TMP); and 3 diacids: adipic acid (AA), sebacic acid (SA) and phthalic acid (PA, undefined isomer). Lactic acid (LA) did not combine with any other monomer as when used in a formulation, it is not in combination with the diol or diacid monomers. BD and PG were the most abundant diols, being present in 42% and 21% of the oligomer forms, respectively. Likewise, AA was the most common diacid, being part of 64% of the oligoester combinations, followed by phthalic acid (8%) and sebacic acid (4%).

Linear and cyclic combinations of AA and BD were common across samples 1 and 3, which indicated a type of blend using poly(1,4-butylene adipate) (PBA), a biopolymer commonly blended with other polyesters to increase their biodegradability and mechanical properties (Debuissy et al., 2016). Various oligoester forms of AA and BD have been reported in other biodegradable food contact materials, being the most common the cyclic form of the tetramer $c[2BD+2AA]$ (Aznar et al., 2019; Canellas et al., 2015; Cariou et al., 2022; Debuissy et al., 2016; E.L. Bradley, 2010). Blends of PLA and PBA with PBAT increase the barrier properties of the resulting material (Bheemaneni et al., 2018; H. Zhang et al., 2013), which we hypothesized is the source of the oligomer combinations containing BD, AA and PA.

When comparing the 3 starch-based materials, two lactic acid oligomers, lin[2LA+C₂H₅] and c[7LA], were the only combinations found across all three samples, suggesting a common PLA component. Ethoxylation (+C₂H₅) of LA oligomers occurs during the migration process in contact with ethanol, meaning no ethoxylated oligoesters would have migrated into the foodstuff (Aznar et al., 2019). Sample 2 showed forms of PG and EG with adipic acid, pointing in this case to the presence of poly(propylene glycol adipate) (PPA) and poly(ethylene glycol adipate) (PEA) as plasticisers (Slobodinyuk et al., 2023; Tang et al., 2020). Only one combination of SA and DEG, commonly used as raw materials in polyester manufacturing (Úbeda et al., 2017; N. Zhang et al., 2020), was found forming a cyclic dimer in sample 3. TMP is a substance used in the production of hyperbranched polyesters, a type of polymer with good properties as coating agents (Zhang et al., 2017). Interestingly, another cyclic dimer containing AA and TMP, could also be hypothesised in sample 3.

Table 1. Compounds hypothesised in the migration of three biopolymers samples (S1, S2 & S3) in food simulants A, B and E. Molecular formula (MF); linear (lin) and cyclic (c) proposed candidates; remarks, main fragments, and their scores (S) obtained by MassFragment. LA: lactic acid, BD: 1,4-butanediol, AA: adipic acid, EG: ethylene glycol, PG: propylene glycol, DEG: diethylene glycol, SA: sebacic acid, PA: phthalic acid, TMP: trimethylolpropane, Rt: retention time, nd: not detected.

Rt	<i>m/z</i> [MNa] ⁺	MF	S1	S2	S3	Candidate	ID level	Remarks/Fragments(scores)
4.37	213.0738	C ₈ H ₁₄ O ₅	E	E	E	lin[2LA+C ₂ H ₅]	2a	PLA oligomer. 161.0450 (S0.5) 158.0256 (S1)
4.4	241.1047	C ₁₀ H ₁₈ O ₅	A	nd	A	lin[BD+AA] (86923-79-7)	1	Polyester oligomer. 202.1232 (S0.5) 147.0637 (S.05)
4.9	367.1349	C ₁₆ H ₂₄ O ₈	nd	A,B,E	nd	c[2EG+2AA]	2a	Polyester oligomer. 346.1604 (S0.5) 174.0846 (S0.5)
4.96	313.1623	C ₁₄ H ₂₆ O ₆	A	nd	A	lin[2BD+AA] (20985-13-1)	1	Polyester oligomer. 155.0724 (S1.5) 111.0431 (S1)
5.00	281.1478	-	E	nd	E	-	-	259.1628, 143.0996
5.08	413.1784	C ₁₈ H ₃₀ O ₉	nd	A	nd	lin[2PG+2AA]	1	Polyester oligomer. 331.1837 (S1) 245.1081 (S1)
5.14	301.2847	C ₁₇ H ₃₇ N ₂ O ₂	nd	nd	A,E	-	-	301.2847, 149.0060
5.54	395.1679	C ₁₈ H ₂₈ O ₈	nd	A,B,E	nd	c[2PG+2AA]	1	Polyester oligomer. 203.0996 (S2) 115.0793 (S2)
5.60	357.1162	C ₁₄ H ₂₂ O ₉	nd	E	nd	lin[4LA+C ₂ H ₅]	2a	PLA oligomer. 315.0782 (S3) 119.0626 (S3)
5.67	383.0966	C ₁₅ H ₂₀ O ₁₀	E	nd	E	c[5LA]	2a	PLA oligomer. 158.0196 (S2) 89.0230 (S2)
5.71	369.1543	C ₁₆ H ₂₆ O ₈	A	nd	A	-	-	329.1606, 201.1153
5.85	441.2094	C ₂₀ H ₃₄ O ₉	A	nd	A	lin[2BD+2AA]	1	Polyester oligomer. 401.2176 (S0.5) 291.1791 (S0.5)
5.87	455.1153	C ₁₈ H ₂₄ O ₁₂	A,B,E	nd	E	c[6LA]	2a	PLA oligomer. 307.1044 (S2) 273.0987 (S2)
5.90	295.1526	C ₁₄ H ₂₄ O ₅	nd	nd	E	c[DEG+SA]	2a	Polyester oligomer. 227.1252 (S2) 203.1207 (S2)
5.92	513.2682	C ₂₄ H ₄₂ O ₁₀	nd	nd	A	lin[3BD+2AA]	1	Polyester oligomer. 458.2516 (S1) 329.1677 (S1)
6.01	567.2431	C ₂₈ H ₃₈ O ₁₂	nd	A,B	nd	lin[2BD+2AA+PA]	2a	Polyester oligomer. 228.1633 (S1) 129.0535 (S0.5)
6.15	527.1379	C ₂₁ H ₂₈ O ₁₄	A,B	A,B	A	c[7LA]	2a	PLA oligomer. 158.0272 (S2) 175.1000 (S3)
6.23	423.1999	C ₂₀ H ₃₂ O ₈	A,B,E	nd	A,B,E	c[2BD+2AA] (CAS 78837-87-3)	1	Polyester oligomer. 311.1474 (S2) 213.1034 (S3)
6.27	739.3167	C ₃₄ H ₅₂ O ₁₆	nd	A,B	nd	c[2EG+2PG+4AA]	2a	Polyester oligomer. 373.1862 (S0.5) 315.1352 (S0.5)
6.30	641.3145	C ₃₀ H ₅₀ O ₁₃	nd	nd	A	lin[3BD+3AA]	1	Polyester oligomer. 547.2840 (S0.5) 431.2293 (S1)

6.31	595.2734	C ₂₈ H ₄₄ O ₁₂	nd	A,B	nd	c[2BD+3AA+EG]	2a	Polyester oligomer. 402.2205 (S2) 301.1345 (S2)
6.49	767.3488	C ₃₆ H ₅₆ O ₁₆	nd	A,B	nd	c[4PG+4AA]	1	Polyester oligomer. 333.1578 (S2) 261.1282 (S2)
6.58	279.1573	C ₁₄ H ₂₄ O ₄	nd	nd	E	c[AA+TMP]	2a	Polyester oligomer. 257.1755 (S0.5) 130.1387 (S0.5)
6.63	443.1689	C ₂₂ H ₂₈ O ₈	A,B	nd	A,B,E	c[2BD+AA+PA]	1	Polyester oligomer. 307.1150 (S2) 221.0857 (S2)
6.67	623.3052	C ₃₀ H ₄₈ O ₁₂	A,B	nd	A,B	c[3BD+3AA]	1	Polyester oligomer. 457.2471 (S3) 429.2100 (S3)
7.03	545.1516	C ₂₁ H ₃₀ O ₁₅	A	nd	nd	lin[7LA]	2a	PLA oligomer. 319.1362 (S1.5)
7.74	256.2641	C ₁₆ H ₃₄ NO	nd	nd	B	-	-	125.9877, 158.0055
7.86	282.2764	C ₁₆ H ₃₇ NO	nd	nd	B	-	-	125.9877, 247.2442
8.36	284.2945	C ₁₈ H ₃₈ NO	nd	nd	B	-	-	125.9865, 158.0121
8.45	319.1955	-	nd	nd	B	-	-	125.9862, 365.2050
10.6	536.1666	-	E	nd	nd	-	-	125.9881, 369.2711

3.2. Synthesis of oligoester standards

3.2.1. Selection of combinations

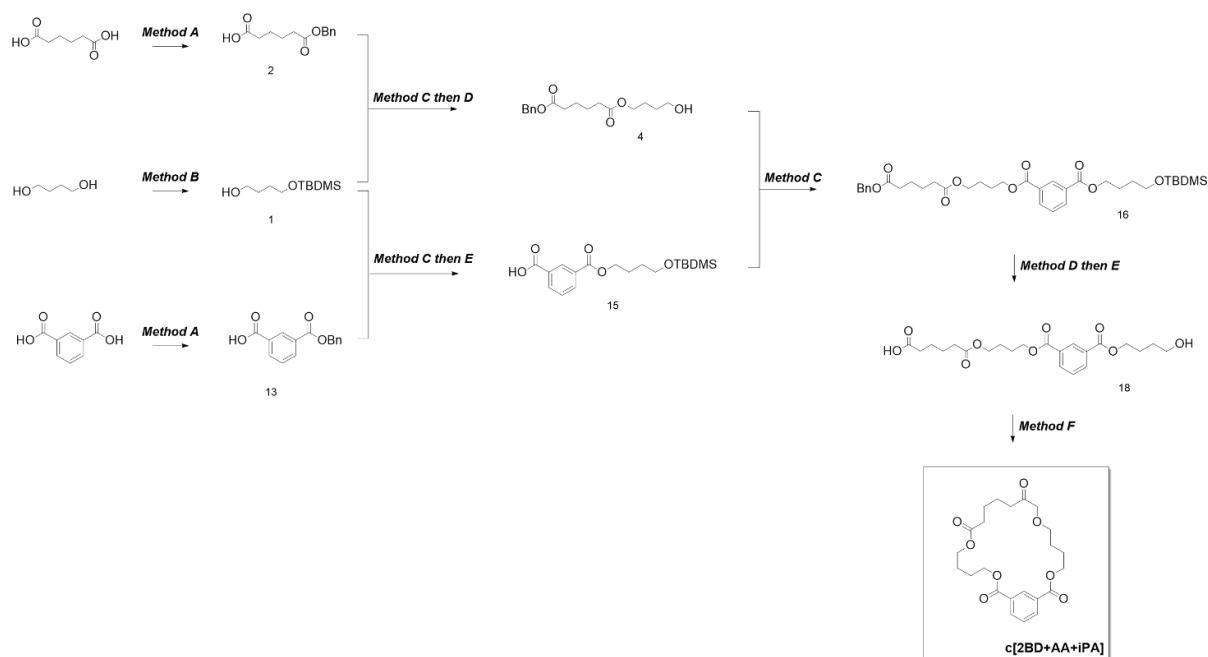
Overall, the lack of NIAS reference compounds represents a major obstacle not only for their use as analytical standard, unequivocally identifying and quantifying them in samples, but also for the need to perform mechanistic and toxicological studies (Shi et al., 2023). When it comes to oligoesters, only a few syntheses have been attempted. Regarding oligoesters containing AA as diacid and BD as diol, only c[2BD+2AA] was available to purchase (LGC standards, Middlesex, UK). However, to the best of our knowledge, no attempt has been performed to synthesize a series of AA and BD oligoesters, so the stepwise synthesis (Figure 3) was seen as an opportunity to obtain not only c[2BD+2AA], but the rest of linear and cyclic oligoesters ranging from 2 to 6 units. As some oligoesters containing EG combined with terephthalic acid (Paseiro-Cerrato et al., 2016) and LA oligomers (Schliecker et al., 2003) have already been synthesized, the efforts were focused in attempting the synthesis of oligomers containing PG as diol (Figure 4). Most of the described oligomeric syntheses involve the use of PA (both isophthalic, iPA, and terephthalic, tPA) as diacid (Cariou et al., 2022; Eckardt et al., 2019; Paseiro-Cerrato et al., 2016; Pietropaolo et al., 2018). However, as none involved the production of c[2BD+iPA+AA], it was decided to attempt it specially because oligoesters containing an aromatic moiety have a higher toxicity potential. In this case, iPA was chosen as PA isomer due to its preferable use to improve barrier properties in biopolymer production (Lee et al., 2022).

3.2.2. Synthesis

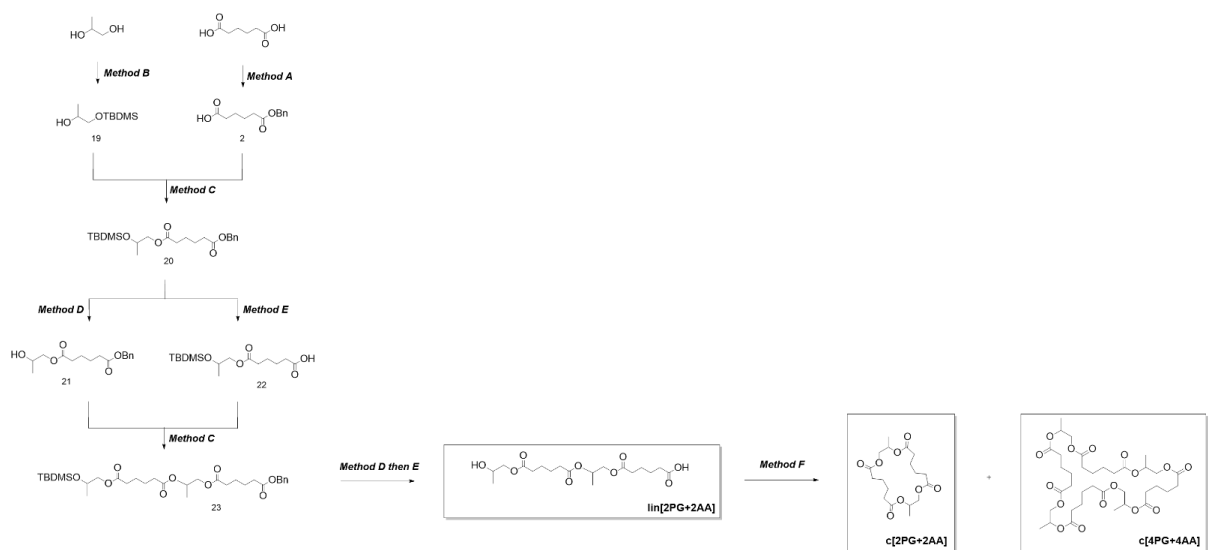
As similarly described by Cariou et al., 2022, a stepwise synthetic approach was applied in this work to prepare new linear or cyclic oligomers based on new diacid monomer such as adipic acid, or new diols units such as butane-1,4-diol or propylene glycol. This robust and well-adaptable multistep synthesis allowed us to yield the expected oligomers with an excellent control of the size and with clean purity for further semi-quantitative analysis.

Our efforts were first dedicated to the preparation of oligoesters derived from butanediol (BD) and adipic acid (AA) (Scheme 2). In comparison to the original synthesis described by Cariou et al., 2022, a slight modification was implemented for the monobenylation reaction

As represented by the following cyclic oligoester $c[2BD+iPA+AA]$ (Scheme 3), the stepwise synthesis allowed satisfyingly the preparation of this expected cyclic 4-units-compound with a global yield of 38% over 11 synthetic steps. This result showed the great efficacy of the synthesis and also that this synthetic strategy was well-adaptable for the production of hybrid oligomers based on varied diacid units (*i.e.* AA and iPA). Finally, our results showed that this stepwise synthesis was very appropriate for the preparation of non-symmetrical oligomers bearing diol unit represented with two differential reactivity of the hydroxyl groups such as a primary and a secondary alcohol (Scheme 4). This type of dual reactivity of the diol partner was applied for the preparation of propylene glycol-based oligomers which are consistent with other NIAS arising from biofilms. Once again, the stepwise synthesis was very convenient for the synthesis of linear and cyclic oligoesters derived from propylene glycol (PG) and adipic acid (AA). Gratifyingly, the linear oligomer $lin[2PG+2AA]$ was cleanly obtained with a global yield of 52% over 9 steps. This compound was used as precursor of the macrolactonization for the most critical step as intramolecular reactions can be favoured over intermolecular ones. While good macrolactonization yields were generally observed (up to 80%), the macrolactonization of $lin[2PG+2AA]$ afforded the expected cyclic tetramer $c[2PG+2AA]$ with a lower yield (73%), because of the concomitant formation of the octamer $c[4PG+4AA]$ (15%) along with the dodecamer $c[6PG+6AA]$ (5 %). The complex mixture components were separated by flash chromatography and only $c[2PG+2AA]$ and $c[4PG+4AA]$ reached adequate quantities for a complete characterisation and further application as reference standards.



Scheme 3- Stepwise synthesis of cyclic tetramer oligoester $c[2BD+iPA+AA]$.



Scheme 4- Stepwise synthesis of linear and cyclic oligoesters derived from propylene glycol (PG) and adipic acid (AA).

3.2.3. Characterisation

Characterisation data of the 11 synthesized oligoesters and their reaction intermediates (1H and ^{13}C NMR, ESI-TOF-HRMS and melting point if applicable) can be found in the supplementary information. Table 2 showcases the chemical purities (w/w) of the final

products. No impurities were identified for c[2BD+2AA] and most of the compounds showed a purity above 98% w/w. The three hydroxy acids from AA and BD; lin[BD+AA], lin[2BD+2AA] and lin[3BD+3AA] were the products with the highest impurities percentage. Most of the impurities involved side esterification reactions forming larger chain oligoesters. One hypothesis is that they might be formed during co-evaporation of the products under heat and vacuum, as continued removal of water can pull the equilibrium of the esterification reaction (Khan et al., 2021).

Table 2. LC-ESI-MS(Orbitrap) purity percentages of the synthesised oligoesters (w/w) and their impurities.

Compound	Purity	Impurities
lin[BD+AA]	29.7 %	lin[2BD+2AA] 54.3%, lin[3BD+3AA] 11.4%, lin[BD+AA+C ₂ H ₅] 3.0%, lin[2BD+2AA+C ₂ H ₅] 1.7%
lin[2BD+AA]	99.3 %	lin[3BD+2AA] 0.7%
lin[2BD+2AA]	81.6 %	lin[4BD+4AA] 13.6%, lin[3BD+3AA] 2.3%, lin[2BD+2AA+C ₃ H ₈] 1.0%, c[2BD+2AA] 0.8%, lin[2BD+AA] 0.6%, lin[BD+AA] 0.1%
c[2BD+2AA]	100 %	-
lin[3BD+2AA]	97.3 %	C ₁₈ H ₂₄ NO 2.3%, C ₁₂ H ₂₈ NO ₃ 0.4%, lin[2BD+AA] 0.1%
lin[3BD+3AA]	98.8 %	C ₃₄ H ₆₀ NO ₁₄ 1.0%, lin[3BD+2AA] 0.1%, lin[2BD+2AA] 0.1%
c[3BD+3AA]	98.6 %	C ₁₆ H ₃₆ NO ₂ 1.1%, 650.3743 m/z [M+NH ₄] 0.3%
c[2BD+AA+iPA]	98.7 %	c[2BD+2AA] 1.3%
lin[2PG+2AA]	98.7 %	lin[3PG+3AA] 0.9%, c[2PG+2AA] 0.4%
c[2PG+2AA]	98.0 %	C ₄₈ H ₂₀ O ₂ 1.0%, C ₄₉ H ₂₂ O ₂ 1.0%
c[4PG+4AA]	98.2 %	C ₄₇ H ₅₆ NO ₂₃ 1.3%, C ₄₉ H ₅₇ O ₆ 0.4%, c[2PG+2AA] 0.1%

3.3. Unequivocal identification and quantification

In order to verify the chemical structure of the oligomer candidates proposed during the identification of migration extracts, MS² spectra of precursor ions were compared with spectra of a 10 mg.kg⁻¹ oligoesters solution. All synthesised oligoesters were identified with the retention time criteria and a minimum of 4 matching MS fragments. The application of the collision energy ramp proved to be a good tool to generate low and high mass fragments (see supplementary information). To the best of our knowledge, this is the first time that lin[BD+AA], lin[2BD+AA], lin[2BD+2AA] c[2BD+2AA], lin[3BD+2AA], lin[3BD+3AA],

c[3BD+3AA], c[2BD+AA+iPA], lin[2PG+2AA], c[2PG+2AA] and c[4PG+4AA] have been unequivocally identified as migrant oligoesters from biopolymer samples.

For quantification purposes, linearity and sensitivity were first determined on standard calibration curves. Gravimetrically prepared solutions of the oligoester standards were analysed by UPLC-MS(QTOF) and the area of the most abundant adduct for each compound ($[M + H]^+$ or $[M + Na]^+$) was obtained with a 10 ppm m/z tolerance. Linearity parameters for each analyte are presented in Table 3. Relative error or bias in the proposed linear range was within the acceptable order of 80 – 120 %. Limits of detection (LOD) were low and ranged from 0.03 to 2.54 $\mu\text{g}\cdot\text{kg}^{-1}$ which is in range of what other authors found for similar compounds (Ubeda et al., 2020). Given that the slope represents the sensitivity of a method towards an analyte, we found differences of up to 30 times when comparing the slopes of c[2BD+AA+iPA] and lin[3BD+3AA]. Although the variation is less pronounced when comparing more chemically-alike oligoesters, it is still relevant and will affect the final concentration values and therefore in the safety compliance of a material. For example, between cyclic oligomers differences in response ranged from 1.1 to 6 times and for linear oligomers from 1.1 to 14 times. Even though differences in the ionization efficiency and transmission between analytes are known to be dependent on several factors (e.g. polarity, size, mobile phase composition), the understanding of the ESI process is still limited (Cech & Enke, 2001; Liigand et al., 2017). Moreover, the lack of oligomer standards makes semi-quantification the only alternative strategy to be applied for reporting purposes (Ubeda et al., 2021).

Table 3. Linearity data of the oligoester standards analysed by UPLC-MS(Q-TOF). Linear range, LOD and LOQ values are expressed in $\mu\text{g kg}^{-1}$. QS: quantifying standard.

QS no.	Compound	Quant m/z	Adduct	Slope	Intercept	R ²	Linear range	LOD	LOQ
Q1	lin[BD+AA]	241.1046	$[M + Na]^+$	10.18	21.87	0.999	6.2 - 1,000	1.87	6.22
Q2	lin[2BD+AA]	313.1622	$[M + Na]^+$	18.02	206.84	0.998	1.5 - 1,000	0.44	1.47
Q3	lin[2BD+2AA]	441.2095	$[M + Na]^+$	12.80	107.87	0.999	1 - 1,000	0.31	1.04
Q4	c[2BD+2AA]	401.2170	$[M + H]^+$	8.73	35.35	0.999	1.7 - 1,000	0.52	1.74
Q5	lin[3BD+2AA]	513.2670	$[M + Na]^+$	24.39	62.91	0.999	0.4 - 150	0.13	0.44
Q6	lin[3BD+3AA]	619.3324	$[M + H]^+$	1.66	-29.39	0.998	8.5 - 1,000	2.54	8.46
Q7	c[3BD+3AA]	601.3219	$[M + H]^+$	7.86	-56.86	0.999	1.1 - 1,000	0.33	1.11
Q8	c[2BD+AA+iPA]	443.1676	$[M + Na]^+$	48.52	477.25	0.999	0.1 - 500	0.03	0.09
Q9	lin[2PG+2AA]	413.1782	$[M + Na]^+$	14.19	29.18	0.998	3.5 - 1,000	1.04	3.46

Q10	c[2PG+2AA]	373.1857	[M + H] ⁺	13.29	126.19	0.998	0.7 - 1,000	0.19	0.65
Q11	c[4PG+4AA]	745.3641	[M + H] ⁺	8.166	-145.625	0.998	50-1,000	0.39	1.28

Migration values of migrant oligoesters ($\mu\text{g}\cdot\text{kg}^{-1}$ of food simulant) are summarised in Table 4. For oligoesters for which a standard was not synthesised, a quantifying standard from Table 3 was chosen according to their structure-type (linear or cyclic), size and free functional groups.

Comparing migration results from the 3 food simulants, simulant E showed the lowest migration values. In addition, due to Tenax different migration profile compared to simulants A and B, simulant E values were similar across all three samples. Despite being considerably higher than in Tenax, migration values in simulant B were smaller than in simulant A, probably due to a lower solubility and the acid effect on the stability of oligoesters (Ubeda et al., 2020).

Considering samples, overall oligoester migration was higher in S2 than in S1 and S3, which were more alike. For example, c[2PG+2AA] was the dominant contributor to the migration from S2, while it was c[2BD+2AA] for both S1 and S3. Concentrations of corresponding linear oligoesters were smaller than their cyclic counterparts but, in both cases, still exceeded the value established by EU/10/2011 for non-listed substances ($10 \mu\text{g}\cdot\text{kg}^{-1}$) except for c[2BD+AA+iPA] (S1, simulant B & S3, simulant E) and lin[4LA+C₂H₅] (S2, Simulant E). As recently stressed by the scientific community, there is hardly any toxicological data available on polyester oligomers (Cariou et al., 2022; Lestido-Cardama et al., 2022) which makes more complicate to perform a proper risk assessment. Recent studies showed androgen receptor (AR) activity of cyclic oligomers from food packaging adhesives (Ubeda et al., 2020) and the potential of PLA oligomers to bioaggregate in the liver, intestine and brain (Wang et al., 2023). In these situations, a *read-cross* approach, such as the threshold of toxicological concern (TTC), could be used (More et al., 2019). TTC assigns a theoretical toxicity class to each compound depending on its chemical structure (Cramer et al., 1976). For this purpose, the software Toxtree v3.1.0 (Ideaconult Ltd., Sofia, Bulgaria) was used. All compounds are divided into three classes according to their theoretical toxicities – low (I), intermediate (II), and high (III) – and are subject to a maximum daily intake above which further *in vitro* testing is required: 1.8, 0.54 and 0.09 mg per person per day, respectively. Moreover, compounds

must not be potential mutagens, carcinogens, organophosphates, or carbamates. Assuming a food consumption of 1 kg/person/day, these intakes can be transformed to maximum recommended migration values. Most of the migrant compounds were listed as Class I, having a maximum recommended migration of 1,800 ng.g⁻¹, which was not exceeded for any of the oligomers. However, four oligoesters; c[2EG+2PG+4AA], lin[3BD+3AA], c[2BD+3AA+EG] and c[2BD+AA+PA], were classified as class III, having a recommended migration limit of 90 ng.g⁻¹. Only c[2BD+AA+PA] did not exceed the limit, making S1 the only biopolymer sample to comply with the legislation under these premisses.

Although TTC values tend to be conservative, underestimation can occur for some compounds (Partosch et al., 2015; Reilly et al., 2019), evidencing the need of toxicological assessment of oligoester migrants regardless of their Cramer class.

Table 4. Concentration of oligomers ($\mu\text{g kg}^{-1}$) with standard deviation (n=3) in three biopolymers migration extracts (S1, S2 & S3) in simulant A, B and E. In brackets in first columns: assigned quantification standard when different (see Table 3 for code). Concentration values in bold represent those surpassing their TTC value.

Compound	Cramer Class	Acetic Acid 3% (v/v)			Ethanol 10% (v/v)			Tenax®		
		S1	S2	S3	S1	S2	S3	S1	S2	S3
lin[2LA+C ₂ H ₅] (Q1)	I	-	-	-	-	-	-	18.7 ± 2.2	28.7 ± 10.1	32.4 ± 13.8
lin[BD+AA]	I	86.9 ± 9.4	-	156.5 ± 4.9	-	-	-	-	-	-
c[2EG+2AA] (Q10)	I	-	801.6 ± 9.1	-	-	1,064.9 ± 5.9	-	-	34.0 ± 8.2	-
lin[2BD+AA]	I	23.6 ± 2.3	-	51.8 ± 2.7	-	-	-	-	-	-
lin[2PG+2AA]	I	-	301.1 ± 3.6	-	-	-	-	-	-	-
c[2PG+2AA]	I	-	692.2 ± 10.0	-	-	1,282.8 ± 5.7	-	-	15.1 ± 4.1	-
lin[4LA+C ₂ H ₅] (Q9)	I	-	-	-	-	-	-	-	3.8 ± 1.7	-
c[5LA] (Q4)	I	-	-	-	-	-	-	19.6 ± 1.1	-	10.2 ± 2.3
lin[2BD+2AA]	I	124.2 ± 9.9	-	201.1 ± 6.9	-	-	-	-	-	-
c[6LA] (Q7)	I	84.6 ± 6.7	-	-	60.5 ± 3.5	-	-	15.8 ± 0.7	-	11.7 ± 2.8
c[DEG+SA] (Q4)	I	-	-	-	-	-	-	-	-	12.4 ± 3.2
lin[3BD+2AA]	I	-	-	165.8 ± 3.3	-	-	-	-	-	-
lin[2BD+2AA+PA] (Q6)	I	-	385.5 ± 9.7	-	-	840.3 ± 20.5	-	-	-	-
c[7LA] (Q7)	I	147.1 ± 12.9	501.8 ± 6.9	59.4 ± 4.2	133.7 ± 9.3	764.1 ± 39.7	-	-	-	-
c[2BD+2AA]	I	374.2 ± 17.5	-	472.1 ± 13.4	814.6 ± 46.8	-	971.3 ± 41.6	46.1 ± 3.9	-	90.4 ± 16.3
c[2EG+2PG+4AA] (Q11)	III	-	251.5 ± 7.0	-	-	688.4 ± 10.5	-	-	-	-
lin[3BD+3AA]	III	-	-	143.5 ± 8.7	-	-	-	-	-	-

c[2BD+3AA+EG] (Q7)	III	-	205.1 ± 2.5	-	-	684.9 ± 17.8	-	-	-	-
c[4PG+4AA]	I	-	65.2 ± 6.2	-	-	273.8 ± 4.8	-	-	-	-
c[AA+TMP] (Q10)	I	-	-	-	-	-	-	-	-	12.1 ± 2.1
c[2BD+AA+PA]	III	2.5 ± 0.8	-	11.6 ± 0.8	11.4 ± 0.7	-	19.5 ± 1.2	-	-	0.5 ± 0.1
c[3BD+3AA]	I	50.8 ± 2.1	-	99.6 ± 1.6	168.8 ± 8.3	-	255.6 ± 6.9	-	-	-
lin[7LA] (Q6)	I	31.4 ± 1.6	-	-	-	-	-	-	-	-
Total linear oligoesters		266.1 ± 20.2	683.6 ± 8.6	724.7 ± 21.0	-	840.3 ± 20.5	-	19.1 ± 2.4	32.5 ± 9.0	32.44 ± 13.8
Total cyclic oligoesters		659.2 ± 39.2	2,517.2 ± 20.8	543.0 ± 16.2	1,140.4 ± 103.0	4,758.8 ± 30.6	1,246.5 ± 45.0	82.0 ± 4.9	49.1 ± 12.2	137.3 ± 26.6
Total oligoesters		925.2 ± 59.3	3,200.9 ± 25.5	1,267.7 ± 35.1	1,140.4 ± 103.0	5,599.1 ± 40.5	1,246.5 ± 45.0	101.1 ± 6.9	81.54 ± 10.1	169.7 ± 14.5

4. Conclusions

In this work, the food safety of three starch-based biopolymer films has been assessed using three food simulants: ethanol 10%, acetic acid 3% and Tenax. A series of non-volatile migrant oligoesters arising mainly from adipic acid, 1,4-butanediol, propylene glycol, ethylene glycol and phthalic acid were detected by UPLC-MS(QTOF). For identification and quantification purposes, 11 linear and cyclic oligoester combinations (lin[BD+AA], lin[2BD+AA], lin[2BD+2AA] c[2BD+2AA], lin[3BD+2AA], lin[3BD+3AA], c[3BD+3AA], c[2BD+AA+iPA], lin[2PG+2AA], c[2PG+2AA] and c[4PG+4AA]) were successfully synthesized by a multi-step approach and characterized by ¹H and ¹³C NMR and LC-MS(Orbitrap). For the first time, the synthesised oligoester standards were unequivocally identified and quantified as migrant oligoesters in biopolymer films, making only one of the three samples compliant according to the TTC approach. The protocol herein described aims to contribute towards the availability of oligoester standards, a prerequisite for very much needed toxicological studies, shedding some light into a grey area in the food packaging sector.

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CRedit authorship contribution statement

D. Rupérez: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Validation; Visualization; Writing - original draft. **M. Rivière:** Conceptualization; Formal analysis; Writing - review & editing. **J. Lebreton:** Funding acquisition; Supervision; Writing - review & editing. **M. Aznar:** Supervision; Writing - review & editing. **F. Silva:** Funding acquisition; Project management; Supervision; and Writing - review & editing. **A. Tessier:** Conceptualization; Supervision; Resources; Writing - review & editing. **R. Cariou:** Conceptualization; Formal analysis; Supervision; Resources; Writing - review & editing. **C. Nerín:** Conceptualization; Funding acquisition; Supervision; Writing - review & editing.

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