ANALYTICAL, *IN VITRO* AND *IN VIVO* METABOLIC STUDIES OF SHORT CHAIN CYCLIC POLYESTER OLIGOMERS (LACTONES) FROM POLYURETHANE LAMINATING ADHESIVES

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ABSTRACT OF THE THESIS

Analytical, *In Vitro* and *In Vivo* Metabolic Studies of Short Chain Cyclic Polyester Oligomers (Lactones) from Polyurethane Laminating Adhesives

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Polyurethane (PU) laminating adhesive for food packaging is often formulated with polyester polyol to impart soft chain segments for the material's flexibility. Polyester polyol synthesis results in undesirable, low molecular weight cyclic oligomer by-products which are commonly observed in Food and Drug Administration (FDA) migration studies. Currently, only a few cyclic polyester migrants were identified and published, many other possible structures are still to be characterized. There is also a lack of information regarding the prevalence and toxicology of these migrants.

From the migration study data reports of 518 industrial laminates, we were able to identify 23 new short chain cyclic polyesters and 4 linear chain esters from PU adhesives. A tabulated summary on the frequency of occurrence for all cyclic polyester migrants that we have identified so far reveals that diethylene glycol adipate cyclic diester (DEG-AA), diethylene glycol isophthalate cyclic diester (DEG-IPA), neopentyl glycol adipate cyclic

diester (NPG-AA) and di-neopentyl glycol adipate cyclic diester (NPG-AA 2+2) to be the migrants for our initial metabolism studies.

We hypothesize that the mammalian non-specific esterase would hydrolyze these cyclic oligomers into their corresponding monoester and monomer precursors.

Our metabolism studies focused on the hydrolysis of cyclic oligomers with porcine liver esterase (PLE). We used a combination of GC-MS and GC-FID with trimethylsilyl derivatization methods for analysis. We also investigated the hydrolysis of cyclic oligomers with human liver S9 fraction, a complex enzyme system which is often used in the Ames test to assess a substance's mutagenic potential. Finally, we collaborated with other labs to analyze for the *in vivo* metabolites of NPG-AA 2+2 from mouse plasma.

The results show stepwise enzymatic conversion of cyclic oligomers to their open ring monoesters followed by the complete hydrolysis to monomers. Different types of cyclic polyester migrants showed different resistance to enzyme hydrolysis under the same test conditions. Experimental parameters such as substrate levels, incubation time, enzyme concentration and surfactant addition were found to influence the degree of hydrolysis. Also, NPG-AA 2+2 was found to rapidly and completely break down in mice. All of the information is useful for future safety assessment investigations.

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1. INTRODUCTION

Multilayer packaging is widely used to improve the shelf-life of food products. By joining two or more plies of materials such as plastic films, papers and/or aluminum foils together, improved barrier, heat sealing and printing properties of the packaging can be achieved. Adhesive lamination is the most common way used in the manufacture of multilayer flexible packaging. It adheres different packaging materials together by solvent, water or solids based adhesives (Marsh et al., 2007). Polyurethane (PU) adhesives are often used in these adhesive lamination processes. They are generally formed by the polyaddition reaction between isocyanates and polyols, in the presence of certain catalysts and additives. The polyols, such as polyester polyols, impart soft segments to PU, making it flexible and elastic.

Recent FDA and EU migration investigations have identified several new migrants from many PU adhesive based laminates. These migrants are short chain cyclic polyesters (Athenstadt et al., 2012; Isella et al., 2013; Shrikhande, 2012) and are considered as the by-products of polyester polyols. Since industrial polyester polyols are produced from polycondensation reaction between diols and dicarboxylic acids (or derivatives), the formation of the cyclic polyesters is inevitable under certain condition. These compounds do not have free hydroxyl groups for further chain elongation and polymerization. They usually have molecular weights less than 1000 da and can easily migrate into food matrices.

Presently, only 21 short chain cyclic polyester migrants have been identified and published. However, there is no indication of the prevalence of these compounds, and many other cyclic polyester migrants still have not been identified because of limited laminate species were involved in previous migration studies.

In the United States, food packaging laminating adhesives are regulated by FDA 21 CFR §175.105 subpart B, §175.1390 and §175.1395. In the European Union, there is no specific legislation for adhesives except (EC) No. 1935/2004 and (EU) No. 10/2011 which regulates food contact materials and articles including adhesives. Nevertheless, neither FDA nor EU is able to provide any limits on short chain cyclic polyesters because safety assessment and quantification methods are lacking.

Currently, only theoretical toxicity evaluation was partially conducted by the open software named Toxtree (Isella et al., 2013), which classify chemicals into high, medium or low levels of concern. This software was developed based on Cramer Classification Scheme, a well-known approach in the European Commission (EC) for structuring chemicals and estimating the Threshold of Toxicology Concern (TTC).

Since no real toxicity test was available for the cyclic polyester migrants, fundamental *in vitro* metabolism studies on these compounds become very important. Based on previous investigations, it is highly probable that they would be enzymatically hydrolyzed after being ingested. Also, the toxicology information of the metabolites can be very valuable in assessing the safety of the migrants.

It was demonstrated early that synthetic polyesters can be biodegraded by microorganism hydrolases and hog liver esterase (Tokiwa et al., 1977). Later *in vitro*

hydrolysis studies on polymeric plasticizers show that ester migrants such as poly (butylene adipate) and poly (propylene adipate) can be broken down to low molecular oligomers in simulated digestive fluid (Castle et al., 1993; Hamdani et al., 2002). It was also proven that low molecular weight aliphatic diester di-2-ethylhexyl adipate (DEHA) undergoes stepwise hydrolysis to adipic acid and 2-ethylhexnol by rat liver, pancreas and small intestine enzymes (Takahashi et al., 1981). Furthermore, a preliminary hydrolysis investigation on short chain cyclic polyester migrants like 1, 4-butane diol, adipic acid, cyclic diester (1, 4 BG diol-AA) and 1, 6 hexanediol, adipic acid, cyclic diester (1, 6 HG-AA) indicated that the migrants can completely break down to their constituent diols and diacids when exposed to porcine liver esterase (PLE) (Shrikhande, 2012).

In our investigation, we gathered all our FDA migration studies on industry manufactured laminates, then summarized all of the identified cyclic polyester migrants and determined the frequency of their occurrence. Based on the evaluation, we were able to select four typical cyclic polyester migrants DEG-AA, DEG-IPA, NPG-AA, NPG-AA 2+2 representing aliphatic, aromatic, mono and dimer type cyclic oligoester migrants respectively, to be the substrates for enzymatic hydrolysis.

We used both PLE and human liver S9 fraction as the enzyme reaction systems because they are commonly used in toxicity studies of migrants. PLE is known for its broad substrate catalytic ability and has been used in many investigations of ester compound metabolism, while human liver S9 fraction is a complex enzyme mixture and has been used in Ames test to assess the mutagenic potential of chemical compounds. The enzymatic hydrolysis products obtained under different reaction conditions were

derivatized and then analyzed by the GC-MS and GC-FID. Calibration curves of the diol and diacid standards were also constructed for quantification of the anticipated hydrolysis products.

The results of the *in vitro* micronucleus test on Chinese hamster cells provided by our cooperating lab showed that NPG-AA 2+2 is cytotoxic in the absence of S9 fraction. This led to our *in vivo* metabolism study of NPG-AA 2+2 on mice. We used GC-MS-SIM to analyze the NPG-AA 2+2 metabolites from mouse plasma.

2. LITERATURE REVIEW

2.1 Laminates and Polyurethane (PU) Adhesives

2.1.1 Manufacture of Laminates

Multilayer packaging plays a vital role in the food packaging market, which can account for 25% of the household food contacting materials consumption (Figure 1). It combines the desired properties from each packaging material layer together to meet the specific needs of the food products.

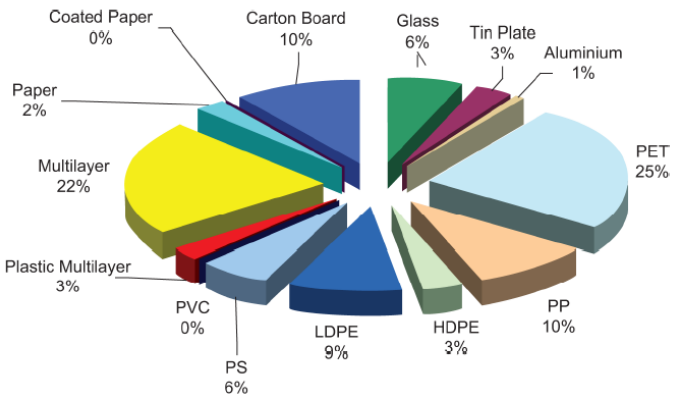


Figure 1: Percent Household Comsuption of Food Contact Packaging Materials

[Adapted from Po ças et al., 2010]

Multilayer food packaging is usually manufactured by either extrusion/co-extrusion or adhesive lamination process. Extrusion and co-extrusion involve melting extrudable resins and forming a thin hot plastic film layer to be conveyed and coated on a plastic, paper or metal foil based substrate. The coated substrate is then pressed with another substrate layer through the counter-rotating rolls to ensure adhesion.

In adhesive lamination, a substrate is coated with an adhesive directly and then mated to the second substrate. It costs less in energy consumption, enables reversible printing and has fewer restrictions on materials' thermal compatibility. Adhesive lamination is also very flexible for short job changes, but it has recycling and migration issues from the adhesive compounds.

Adhesive lamination can be further divided into dry bonding, wet bonding, solventless and UV /EB curing laminating processes based on the adhesive types and application technique. PU is the most widely and commonly used adhesive in these processes because it has excellent wetting ability and can form hydrogen bonds, covalent bonds and van der Waals forces for good adhesion (Table 1).

Table 1: General Adhesive Lamination Technology Comparison

[Adapted from T.E. Rolando]

Lamination	Dry Bond	Wet Bond	100%Solid (Solventless)	Energy (UV/EB) Curable
Typical Adhesives	PU Dispersion Acrylic Emulsion	PU dispersion Acrylic Emulsion	PU dispersion	Acrylate/ Methacrylate monomers/oligomers
Equipment	Gravure, drying oven, heated nip	Gravure, drying oven, heated nips	Heated rollers, heated nips	UV lamp/ Electron beam system

2.1.2 PU Laminating Adhesives for Food Packaging

PU was discovered by Bayer et al. in 1937. It is a class of polymers that has been used in various industries such as textile, footwear, paints, coating, furniture, construction and packaging.

In industry, PU is generally obtained from the polyaddition reaction between isocyanates and polyols in the presence of certain catalysts and additives. Different isocyanates and polyols will give PU different thermoplastic, elastomeric and thermoset properties (Engels et al., 2013). Toluene diisocyanate and methylene diphenyl diisocyanate are the two most representative isocyanate raw materials for PU. They can form the hard segment of the polymer chain to enhance the crystallinity and hardness of PU. Polyols mainly come from two classes of compounds: the polyether polyols and the polyester polyols. They usually form the soft segment and contribute to the tensile strength and elongation (Figure 2). Excess of diisocyanates reacting with polyols will yield isocyanate-terminated PU prepolymers, which can react with many active hydrogen containing compounds (Lay et al., 1994).

PU adhesives can be used in one-component or two-component systems. One component systems contain either completely reacted high molecular weight PU or reactive prepolymers with free NCO groups. The two-component systems require mixing and reacting a low molecular weight polyol with a low molecular weight isocyanate or prepolymer; it is widely used where fast cure speeds are critical or a volatile blocking agent would influence the adhesive properties.

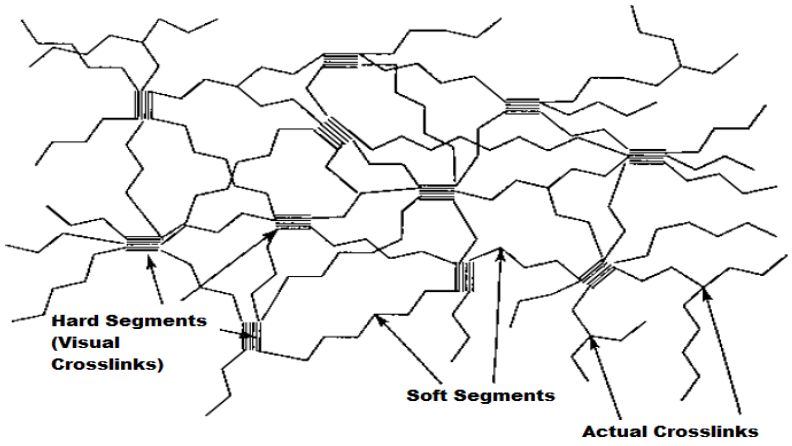


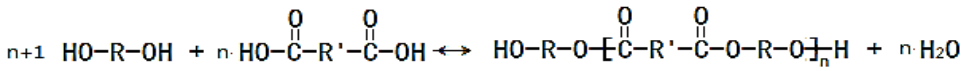
Figure 2: PU Structure [Adapted from Lay et al., 1994]

2.1.3 Polyols in PU Adhesive Manufacture

The very early polyols used in PU adhesives were almost entirely polyesters. In 1958, polyether polyols started to take the market and now they have become the major polyols used in PU because of the advantages of cost and hydrolysis resistance. Polyether polyols are formed in an anionic polyaddition mechanism: open ring monomeric epoxides are polymerized by transferring an anionic charge that initiated from a starter alcohol in strong base.

Polyester polyols are the second widely used polyols in PU. Their polar ester groups guarantee specific adhesion to many materials and they are especially valuable in two component PU systems (Oertel et al., 1985). Compared to polyether polyols, polyester polyols are more heat stable and more resistant to oxidation.

Polyester polyols are produced by polycondensation reaction between diols and dibasic acids:



Ethylene glycol (EG), DEG, triethylene glycol (TEG), 1,2-proplyene glycol (1, 2 PG) and dipropylene glycol (DPG) are the common diols used in polyester polyol production. They are produced from ethylene oxide or propylene oxide and are relatively cheaper. Di-primary glycols such as 1,4 butanediol (1,4 BG), 1,6 hexanediol (1,6 HG), NPG and1,3 butanediol (1,3 BG) are also taking larger shares of the market because they can incorporate some desired properties such as hydrolytic stability to polyester polyol. Branched triols and some long chain trihydroxy compounds are also used in some rare cases.

Adipic acid (AA) is the most important aliphatic type diacid source used for polyester polyol manufacture. It contributes to the soft and elastic PU foam formation (Fink, 2013). Oxalic acid, succinic acid and glutamic acid are less important short chain aliphatic diacids, because the polyesters made from them show inferior hydrolysis stability, whereas the long chain aliphatic diacids such as azaleic acid (AzA), sebasic acid (SeA) and tricarboxylic acids have a price disadvantage. Phthalic acid is also a relatively economical commercial diacid component, which is obtained from phthalic anhydride oxidation. It is aromatic and contributes to the PU rigidity. Isophthalic acid (IPA) and terephthalic acid (TPA) have characteristics similar to phthalic acid and are used for the same purpose. Occasionally, phthalic anhydride may be used in polyesters and only as part of the acid component for modification purpose.

2.2 Short Chain Cyclic Polyester Migrants

2.2.1 Short Chain Cyclic Polyester Formation

The polyester polyol polymerization process requires continuous removal of water to push the equilibrium towards the ester side. The molecular weight of the polymers keeps increasing and the system becomes very viscous from the water removal; diols are also lost at the same time and it becomes more and more difficult to continue with the polymerization because of mass transfer limitation. On the other hand, formation of short chain cyclic polyesters will be favored because of their perfect stoichiometry and they do not require constant water removal (Brunelle, 2003).

Almost all polyester polyols contain a certain quantity of low molecular weight cyclic polyesters (Ionescu, 2005). These compounds do not have free hydroxyl groups for further polycondensation and polyaddition reactions (Figure 3). They cannot react with diisocyanates to form both PU prepolymers and high molecular weight PU. Therefore, short chain cyclic polyesters are generally considered as inevitable undesirable by-products in industries.

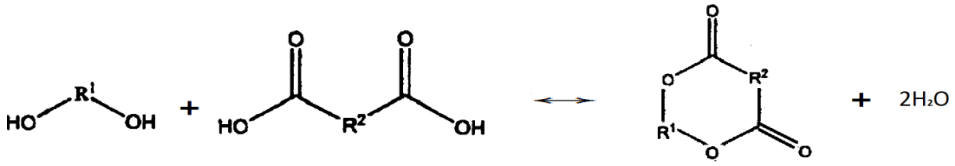


Figure 3: Short Chain Cyclic Polyester Formation

2.2.2 Identification and Analysis of Cyclic Polyester Migrants

Since most of the cyclic polyesters have molecular weight less than 1000, they are high potential migrants from PU adhesive based laminates. Currently, 21 cyclic polyesters

have been identified from migration studies in Europe and United States. Twelve of them are based on different combinations of relatively common and cheap polyester polyol raw materials such as EG, DEG IPA and AA (Table 2). Many theoretically existing cyclic polyester migrants that are based on other diols and diacids have not been identified yet, because the laminates involved in previous migration studies were limited in quantity and variety. Also, there is also a lack of evaluation on the occurrence of each cyclic polyester compound to indicate which migrants need to be of concern most for the primary safety assessment.

GC-MS (CI or EI), ultra performance liquid chromatography coupled to quadrupole time-of-light mass spectrometry (UPLC-Q-TOF/MS) and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS) are the current analytical methods for identifying the short chain cyclic polyester migrants from laminates. Other studies also used LC–MS to identify similar structure cyclic polyester migrants from can coatings, PET bottles and PET trays (Kim et al., 2012; Nasser et al., 2005; Schaefer et al., 2004)

MALDI-TOF/MS is a soft ionization technique in mass spectrometry and mainly used in analysis of large and non-volatile molecules such as protein, peptides and DNA. It is based on chemical matrix in the form of small, laser-absorbing organic molecules in large excess over the analytes (Hillenkamp et al., 2013). MALDI-TOF/MS can generate molecular ions more than 10000 Da with little fragmentation. However, it is not very suitable for identification of smaller cyclic polyester migrants and quantitative analysis for these compounds.

Table 2: Identified Short Chain Cyclic Polyester Migrants from Laminates

Identified Cyclic Polyester	Formula	M.W.	Analytical Method	Reference
DEG-AA	$C_{10}H_{16}O_5$	216	GC-MS(EI)	Shrikhande, 2012 Athenstadt et al., 2012 (F đix et al., 2012)
			UPLC-Q-TOF/MS	Isella et al., 2013
DEG-AA 2+2	$C_{20}H_{32}O_{10}$	432	GC-MS(EI)	Shrikhande, 2012 Athenstadt et al., 2012
			MALDI-TOF/MS	Lawson et al., 2000)
DEG-IPA	$C_{12}H_{12}O_5$	236	GC-MS(EI)	Athenstadt et al., 2012 Shrikhande, 2012
EG-AA	$C_8H_{12}O_4$	172		
DEG-IPA 2+2	$C_{24}H_{24}O_{10}$	472	GC-MS(EI)	Athenstadt et al., 2012
DEG-IPA-DEG-AA	$C_{22}H_{28}O_{10}$	452		
EG-IPA 2+2	$C_{20}H_{24}O_8$	384		
EG-AA 2+2	$C_{16}H_{24}O_8$	344		
EG-IPA-EG-AA	$C_{18}H_{20}O_8$	364		
DEG-AA-EG-AA	$C_{18}H_{28}O_9$	388	GC-MS(CI)	
DEG-IPA-EG-IPA	$C_{22}H_{20}O_9$	428		
DEG-AA-EG-IPA	$C_{22}H_{24}O_9$	408		
1,4 BG-AA	$C_{10}H_{16}O_4$	200	GC-MS(EI)	Shrikhande, 2012 F đix et al., 2012
			UPLC-Q-TOF/MS	Isella et al., 2013
1,4 BG-AA 2+2	$C_{20}H_{32}O_8$	400	GC-MS(EI)	Shrikhande, 2012
			UPLC-Q-TOF/MS	Isella et al., 2013
Heptaethylene glycol-AzA	$C_{11}H_{18}O_4$	214	UPLC-Q-TOF/MS	Isella et al., 2013
Heptaethylene glycol-AzA 2+2	$C_{22}H_{36}O_8$	428		
1,6 Hexanediol-AA	$C_{12}H_{20}O_4$	228	GC-MS(EI)	Shrikhande, 2012
NPG-AA	$C_{11}H_{18}O_4$	214		
NPG-AA 2+2	$C_{22}H_{36}O_8$	428		
DEG-PA	$C_{12}H_{12}O_5$	236		
DEG-PA 2+2	$C_{24}H_{24}O_{10}$	472		

Both GC-MS and UPLC-Q-TOF/MS couple chromatography separation techniques with mass spectrometry for analysis. UPLC is an advanced version of HPLC in which a column is packed with smaller particles allowing operation at higher pressures. This provides better resolution, sensitivity, separation efficiency and faster speed than HPLC (Nováková, 2006). However, UPLC is relatively expensive and less available in industry for daily quality control analysis. UPLC and HPLC analyses are very suitable for analyzing non-volatile compounds, but for smaller volatile or semi-volatile cyclic polyesters with stronger migration potential, GC analysis would be a better choice. For mass spectrometry, TOF/MS is fast and does not have upper limit to the m/z scale theoretically, but a high vacuum system is required for high accuracy and resolution (Glish, 2003). Quadrupole mass spectrometer is cheaper and more widely used in industry.

2.3 Regulation and Safety Assessment

2.3.1 Current Regulation Regarding PU Adhesives

In United States, PU adhesives are allowed to be used in food packaging under defined conditions in compliance with FDA regulations. Substances for use only as components of PU adhesives are regulated in 21 CFR §175.105 subpart B (CFR, 2013). Further application of PU adhesives in food contact laminates should comply with FDA 21 CFR §177.1390 for the use at temperature of 250 °F and above (CFR, 2013), or §177.1395 for temperatures between 120 °F and 250 °F (CFR, 2013)

There is no specific legislation for adhesives in the EU. However, plastic materials and articles that are printed, coated or held together by adhesives should comply with

Regulation (EC) No. 1935/2004 and (EU) No. 10/2011. Their constituents must not be transferred into food in quantities that may harm human health. Recently, the EU initiated a MIGRESIVE Project to support legislation and standardization related to adhesive migration in food packaging materials (Stormer et al., 2009)

Neither FDA nor EU has any specific limitations regarding short chain cyclic polyester migrants because information about the risk and safety of these compounds is still insufficient to support any legislation.

2.3.2 FDA Guidance for the Industry

According to FDA definition in 21 CFR 170.3(e) (1), packaging materials that contact and are reasonably expected to migrate into food can be classified as "food additives" unless those substance are exempted in section 201(s) of the act (21 USC §321) (CFR, 2013). In addition, there is a "no migration exemption" for substances which cannot be detected in a migration study with suitable detection limits, usually either 10 parts per billion (ppb) or 50 ppb (Heckman, 2001).

If a substance detected as a reasonable food component is not Generally Recognized as Safe (GRAS) or prior-sanctioned, its use for food-contact applications must be authorized by a Food Contact Notifications (FCN) filed according to Section 409(h) (1) (21 USC §348) and allowed to become effective, an FDA food additive regulation issued in response to a food additive petition (FAP), or a Threshold of Regulation exemption granted under 21 CFR 174.6 and 170.39 (Heckman, 2005).

In particular, FDA outlines toxicology recommendations to guide the industry for preparation of FCN for Food Contact Substances (FCS) submission. It involves

estimating dietary exposure and performing the recommended corresponding toxicity tests (Table 3). For FCS with higher dietary exposure, more toxicity tests are usually recommended. If the FCS dietary exposure is above 1 ppm, discussion with FDA are recommended to determine if a FAP submission is more appropriate than FCN.

FDA also outlines the chemistry recommendations to guide preparation of premarket FCN, FAP for FCS submissions (FDA, 2007). The migration testing and analytical methods are specifically described in sections of experimental design, test solutions characterization and data reporting, analytical methods, migration database, and migration modeling. More details such as the condition of use, food simulants selection, consumer exposure calculations are also covered in this guidance.

Table 3: Toxicity Tests Recommendation for FCS

[Adapted from FDA, 2002]

FCS Dietary Exposure	**Toxicity Test Recommendation for FCN Submission**
<1.5 µg/person/day (<0.5 ppb))	No toxicity tests needed. Available mutagenic or carcinogenic potential information from literature should be provided.
>1.5 µg/person/day and <150 µg/person/day (>0.5ppb and < 50ppb)	Short-term genetic toxicity tests like Ames test and *in vitro* cytogenetic test in mammalian cells or mouse lymphoma assay are recommended.
>150 µg/person/day and <3000 µg/person/day (>50 ppb and <1ppm)	Genotoxicity assays (Ames test, In vitro cytogenetic test or in vitro mouse lymphoma assay and *in vivo* micronucleus test) Two 90-day subchronic studies, one in a rodent species and the other in a non-rodent species. Additional appropriate studies

2.3.3 Current Safety Assessment Status

Since most of the cyclic polyester migrants have just been recently identified, their toxicity is generally unknown. Only theoretical toxicity evaluation from Toxtree software was available for some of the migrants (Table 4). This software is based on Cramer Classification Scheme, a widely used approach for classifying and ranking chemicals

according to their expected level of oral systemic toxicity, which differentiates chemicals into three classes (Lapenna et al., 2011):

• Class I: Substances have simple chemical structure and efficient metabolism modes, suggesting a low order of oral toxicity.

• Class II Substances contain structural features which are suggestive of toxicity between substances of class I and class III.

• Class III Substances permit no strong initial presumption of safety or may even suggest significant toxicity according to their structure, or substances possess reactive functional groups.

Table 4: Short Chain Cyclic Polyester Migrants Toxtree Evaluation

[Adapted from Isella et al., 2013]

Polyester Migrants	EG-AA	1,4BG-AA	1,4BG-AA 2+2	Heptaethylene glycol-AzA	Heptaethylene glycol-AzA 2+2
Class	II	I	I	I	I

Patlewicz et al. (2008) evaluated the implementation of the Cramer classification scheme in the Toxtree software. Their results showed that the software overall performed well on the systematic evaluation of Cramer structural classes, but also several common food components and normal body constituents were misclassified. Therefore, current software evaluation may not be adequate to support the risk and safety assessments of short chain cyclic polyester migrants.

2.4 Previous Metabolic Investigations of Ester Migrants

Even though there has been very limited toxicity research on short chain cyclic polyester migrants, previous *in vitro* hydrolysis investigations on similar ester migrants provided a clue about how the cyclic polyester migrants may be metabolized.

Tokiwa et al. (1977) first demonstrated that aliphatic synthetic polyesters such as poly(ethylene adipate), poly(ethylene azelate), poly(ethylene sebacate) can be hydrolyzed by microorganism lipase and hog liver esterase, while aromatic synthetic polyesters like poly(tetramethylene terephthalate) and poly(2,2-dimethyl-trimethyelene isophthalate) cannot be degraded under the same test conditions. This indicates that aromatic polyesters are more resistant to hydrolysis than aliphatic polyesters. The specific hydrolysis products could not be determined by measuring the total organic carbon concentration in this study, except poly(ethylene adipate) which was confirmed to break down to ethylene glycol and adipic acid by GC analysis (Figure 4).

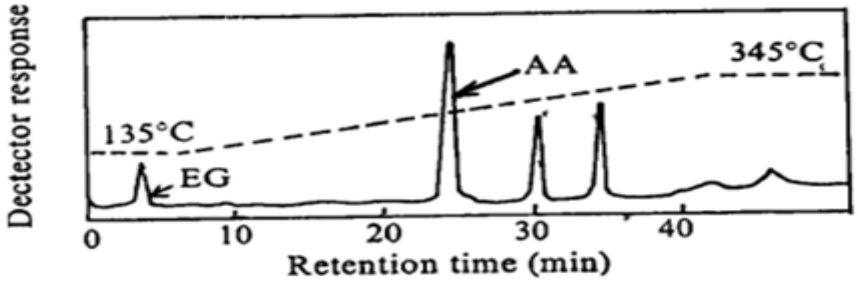


Figure 4: Hydrolysis of Poly (ethylene adipate) by Microbial Esterase

[Adapted from Tokiwa et al., 1977]

Later Castle et al. (1993) exposed poly(butylene adipate), poly(propylene adipate) and their corresponding low molecular weight oligomers to *in vitro* simulated gastric and

intestinal fluid hydrolysis and analyzed the loss of parent compounds by High Pressure Size Exclusion Chromatography (HPSEC). Results showed that the esters were only partially broken down with lower molecular weight oligomers being more susceptible. GC analysis found no significant increase of free monomer hydrolysis products such as adipic acid.

Similarly, Hamdani et al. (2002) exposed poly(1, 2-propylene adipate) to simulated saliva, gastric and intestinal fluid hydrolysis. Their HPSEC analysis showed that parent plasticizer compound completely disappeared and low molecular weight oligomers were formed in simulated intestinal fluid hydrolysis, while the hydrolysis was insignificant under simulated saliva and gastric fluid without any enzyme addition. Adipic acid monomer was also not found in their GC analysis, although the NMR analysis result shows that the pancreatin selectively catalyzed the cleavage of the primary alcohol ester linkage. The formation of monomer products was speculated from a nonselective cleavage of both primary and secondary ester bonds by an enzyme such as a nonspecific esterase.

Later investigations involved the use of human salivary esterase to break down low molecular weight phthalate diesters such as di-n-butyl phthalate (DBP) and di-2-ethylhexyl phthalate (DEHP) (Niino et al., 2003). The GC-MS and HPLC results showed that the diesters can break down to monoesters, but free phthalic acid was not formed (Figure 5). Monoester phthalates are carcinogenic and contribute greatly to the toxicity of those diester phthalates.

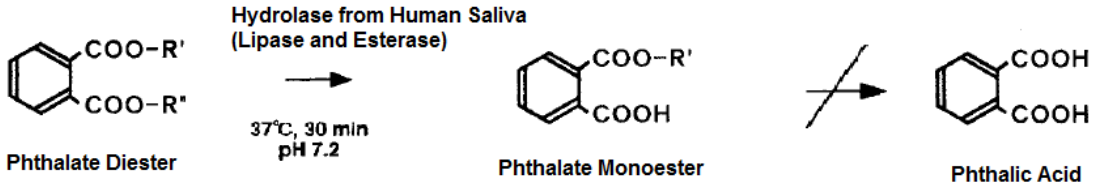


Figure 5: Hydrolysis of Phthalate Esters by Human Saliva Hydrolase

[Adapted from Niino et al., 2003]

Saito et al. (2010) also studied enzymatic hydrolysis of eight structurally diverse phthalic acid esters by porcine and bovine pancreatic cholesterol esterase, including DBP and DEHP. According to their HPLC and thin layer chromatography (TLC) analyses, only monoesters were found as the final products, and no phthalic acid monomer was detected (Figure 6). It was suggested that the partial hydrolysis is due to the anionic charge of the free carboxyl group that inhibits the formation of an enzyme-substrate complex.

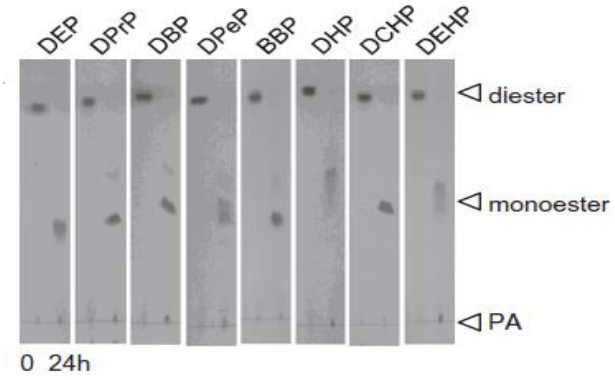


Figure 6: Hydrolysis of Phthalate Esters by Porcine Pancreatic Esterase

[Adapted from Saito et al., 2010]

For adipic acid based diesters such as di-(2-ethylhexyl)adipate (DEHA), investigation shows that its hydrolysis to adipic acid and 2-ethylhexanol by rat liver, pancreas and small intestine enzymes involved the formation of intermediate

mono-(2-ethylhexyl)adipate (MEHA), which can be more rapidly hydrolyzed to the monomers (Takahashi et al., 1981) (Table 5). The investigation also involved DEHP hydrolysis as the comparison, and the results showed that the total reaction rate of DEHA was ten times of DEHP. To further determine the metabolites of DEHA, Loftus et al., (1993) orally administered 46 mg deuterium-labeled DEHA to six male volunteers and analyzed for DEHA and its metabolites in blood samples taken from the volunteers at 0.5, 1, 2, 3, 4, 5, 6, 8, and 12 hours after dosing. The parent compound was not found and deuterated 2-ethylhexanoic acid was the only measurable DEHA-related compound in the plasma. Traces of 2-ethylhexanol (EH) were also detected but not quantifiable.

Table 5: *In Vitro* Hydrolysis of Di-(2-ethylhexyl)adipate and Mono-(2-ethylhexyl)adipate by Rats Tissue Enzymes

[Adapted from Takahashi et al., 1981]

Substrate	Enzyme Source	Product (nmol/mg protein/min)		
		AA	MEHA	Total
DEHA	Liver	16.7	1.0	17.7
	Pancreas	17.1	41.7	58.8
	Intestine	14.8	Trace	14.8
MEHA	Liver	86		86
	Pancreas	57.9		57.9
	Intestine	251.4		251.4

Hooker et al. (2003) studied the enzymatic catalyzed hydrolysis of PET cyclic trimer. Although they used cutinase (a microbial enzyme which specifically acts on carboxylic ester bonds) instead of mammalian esterases, their analysis results still are very useful for proposing the potential metabolites of short chain cyclic polyester migrants because the PET trimer has lactone structure similar to the cyclic migrants. According to their HPLC analysis results, ring cleavage of the PET cyclic trimer resulted in three significant hydrolyzed products: terephthalic acid (TA), monohydroxyethylene terephthalate (MEHT) and bis (hydroxyethyl) terephthalate (BHET). TA and MHET were much more predominant than BHET (Figure 7). Use of a surfactant such as Triton X-100, vigorous agitation and higher concentrations of enzyme facilitates more complete hydrolysis. Since the enzyme reaction is very fast, it can be difficult to detect some possible intermediates such as the open ring linear trimer and linear dimer (Figure 8).

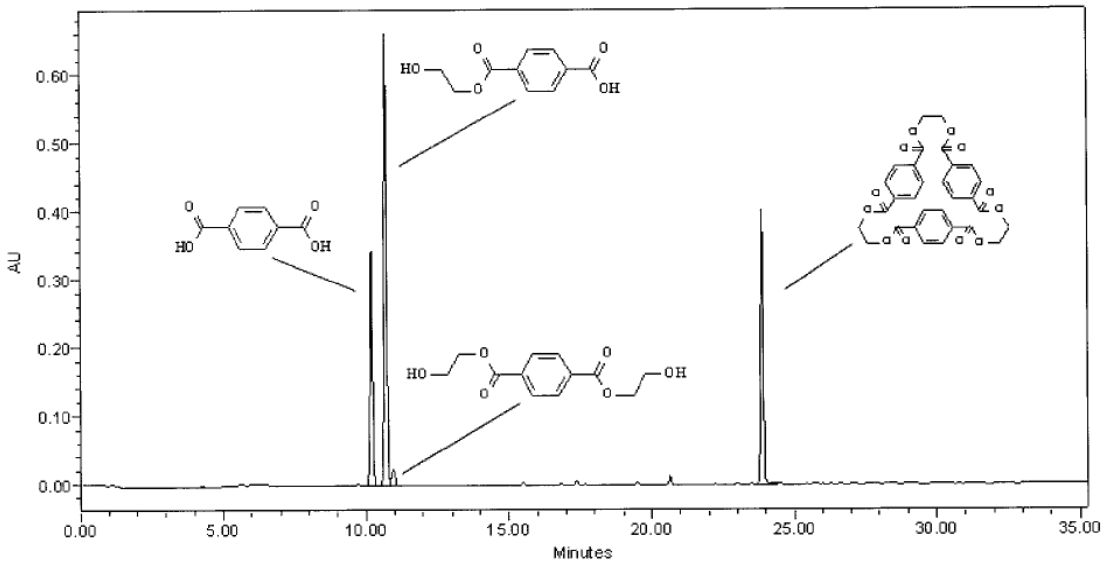


Figure 7: Hydrolysis of PET Cyclic Trimer by Cutinase

[Adapted from Hooker et al., 2003]

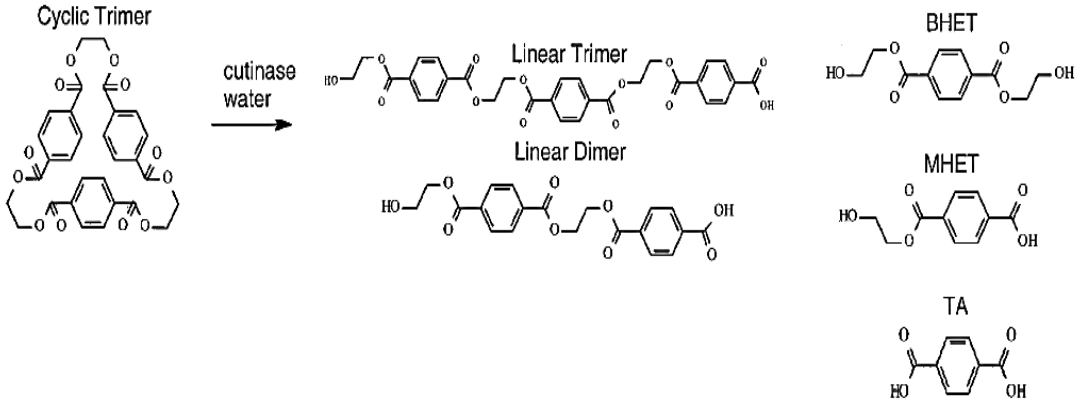


Figure 8: Suggested Hydrolysis Products of PET Cyclic Trimer

[Adapted from Hooker et al., 2003]

A preliminary investigation by Shrikhande (2012) exposed short chain cyclic polyester migrants such as 1,4 BG-AA and 1,6 HG-AA to PLE hydrolysis and the analysis incorporated TMS derivatization method with GC-MS. Results showed that all of the short chain cyclic polyester migrants completely decomposed to their corresponding diols and diacids without any formation of intermediates. This investigation was lacking in authentic short chain cyclic polyester standards for the hydrolysis study and the cyclic polyesters from laminate extracts were in trace amount. Also, the tests were all conducted under one condition at 37 ℃ for 1 hour with the same PLE concentration. The design of the experiment was inadequate for the detection of any transitional or rapid formation of an intermediate hydrolysis product.

2.5 PLE and Human Liver S9 Fraction

Porcine Liver Esterase (PLE) and Human Liver S9 fraction are the ideal enzyme reaction systems to investigate the hydrolysis of short chain cyclic polyesters.

PLE is known for its broad substrate catalytic ability and has been used in many investigations of enzymatic metabolism of ester compounds. It is a serine type esterase and can hydrolyze wide range of ester structures. PLE has been used as the catalyst for the hydrolysis of cyclic meso diesters to prepare chiral half-esters because of its enantioselectivity (Moorlag et al., 1990). PLE and the other mammal nonspecific carboxylesterases play a key role in the detoxification and metabolism of various exogenous and endogenous compounds (Satoh et al., 1998).

Human liver S9 fraction is the supernatant fraction obtained by the centrifugation of human liver homogenate at 9000g for 20 minutes (Hakura et al., 2001). It contains the cytosol and microsomal parts of the cells after removal of larger fragments of nuclei and mitochondria. Human liver S9 fraction is a complex system that involves different enzyme activities including Cytochrome P450 (CYP450) mixed function oxidases, transferases and nonspecific carboxyl esterases. These enzymes are highly involved in xenobiotics metabolism: CYP450 is the primary enzyme involved in the phase I oxidation, while the transferases are closely related with phase II reactions such as glutathione conjugation and glucuronidation. At the same time, hydrolysis reactions catalyzed by enzymes such as epoxide hydrolyses and carboxyl esterases are also active (Parkinson, 2001). Thus, the S9 fraction has been used as the major *in vitro* experimental protocol for various metabolic investigations. For example, the S9 fraction is commonly used in the Salmonella/microsome bacterial mutagenicity test (Ames test) to assess a chemical compound’s mutagenic potential.

3 RESEARCH HYPOTHESES AND OBJECTIVES

3.1 Hypothesis

Based on the previous *in vitro* hydrolysis investigations of ester migrants (Section 2.4), we hypothesize that the cyclic polyesters ingested with food as packaging borne migrants can be stepwise metabolized to open ring monoesters, diols and diacids as depicted in Figure 9.

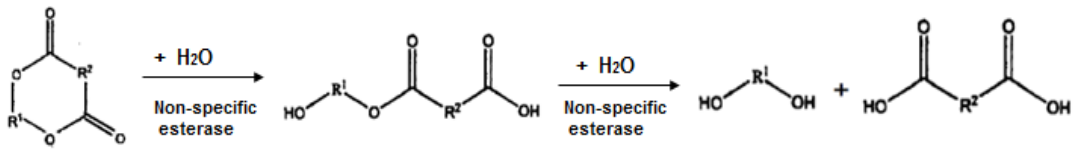


Figure 9: Proposed Hydrolysis Process of Cyclic Short Chain Cyclic Polyesters

We anticipate that aliphatic short chain cyclic polyester migrants will break down more easily than aromatic and dimer type migrants under the same test conditions.

We also hypothesize that experimental parameters such as the use of surfactant, enzyme concentrations and incubation time will influence the hydrolysis rate and the final products formed.

3.2 Objectives

The overall objectives are divided into three parts. First, sort out the reports on migration studies we conducted in the past for our lab projects and determine the occurrence of cyclic polyester migrants in laminates for food applications. Second, select the most representative cyclic polyester migrants for *in vitro* enzymatic hydrolysis studies. Third, analyze the *in vivo* metabolites of the cyclic migrant of interest, NPG-AA 2+2 from mouse plasma.

Specific Tasks includes:

• Summarize the newly identified short chain cyclic polyester migrants

• Record and determine each cyclic polyester migrant's frequency of occurrence from 518 reports on laminate migration studies and select the ones for hydrolysis study.

• Use GC-MS (EI) to identify the hydrolysis products of selected short chain cyclic polyester migrants by PLE.

• Evaluate the influence of experiment parameters such as incubation time, surfactant addition and PLE concentration on the cyclic polyester migrant enzymatic hydrolysis.

• Use GC-FID to build the calibration curve for quantitating possible diol and dibasic acid monomer metabolites.

• Use GC-MS (EI) to analyze the hydrolysis products of selected short chain cyclic polyester migrants by human liver S9 fraction.

• Use GC-MS (EI) to analyze for the *in vivo* metabolites of cyclic NPG-AA 2+2 from mouse plasma.

4 EXPERIMENTAL

4.1 Experimental Overview

To achieve the goals we outlined in the previous section, our experiment was divided into three sections:

Section 1: Thoroughly review accumulated data reports of FDA migration studies on laminates that we had conducted over the past several years to summarize the newly identified short chain oligoesters by GC-MS analysis. Tabulate the frequency of occurrence for all short chain polyester migrants and select the most prevalent and representative ones as candidates for our metabolic studies.

Figure 10 shows the general experimental sequence and organization of our previous migration studies.

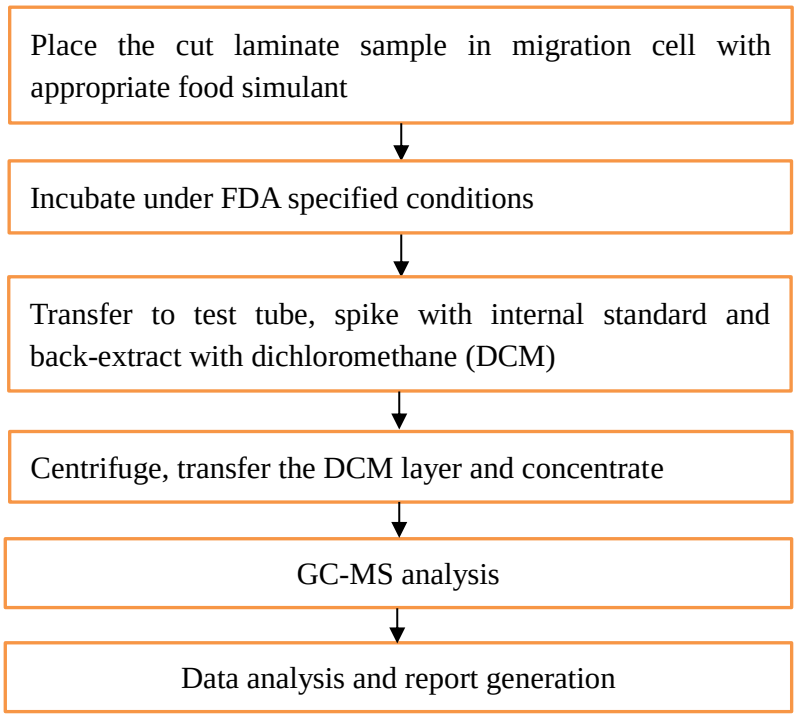


Figure 10: Flow Chart of Experimental Section 1

Section 2: Investigate the *in vitro* enzymatic hydrolysis of the selected migrants from Section 1: DEG-AA, DEG-IPA, NPG-AA 2+2 (synthesized authentic standards available) and NPG-AA (from actual laminate extracts) (Figure 11).

This section can be further divided into PLE hydrolysis and human liver S9 fraction hydrolysis. Since the human liver S9 fraction system contains many different enzyme species and is too complex to work with in the presence of other migrants from the actual laminate extract, only synthesized authentic cyclic polyester standards were tested with it. Calibration curves of potential metabolites such as diacids and diols were also included in this section.

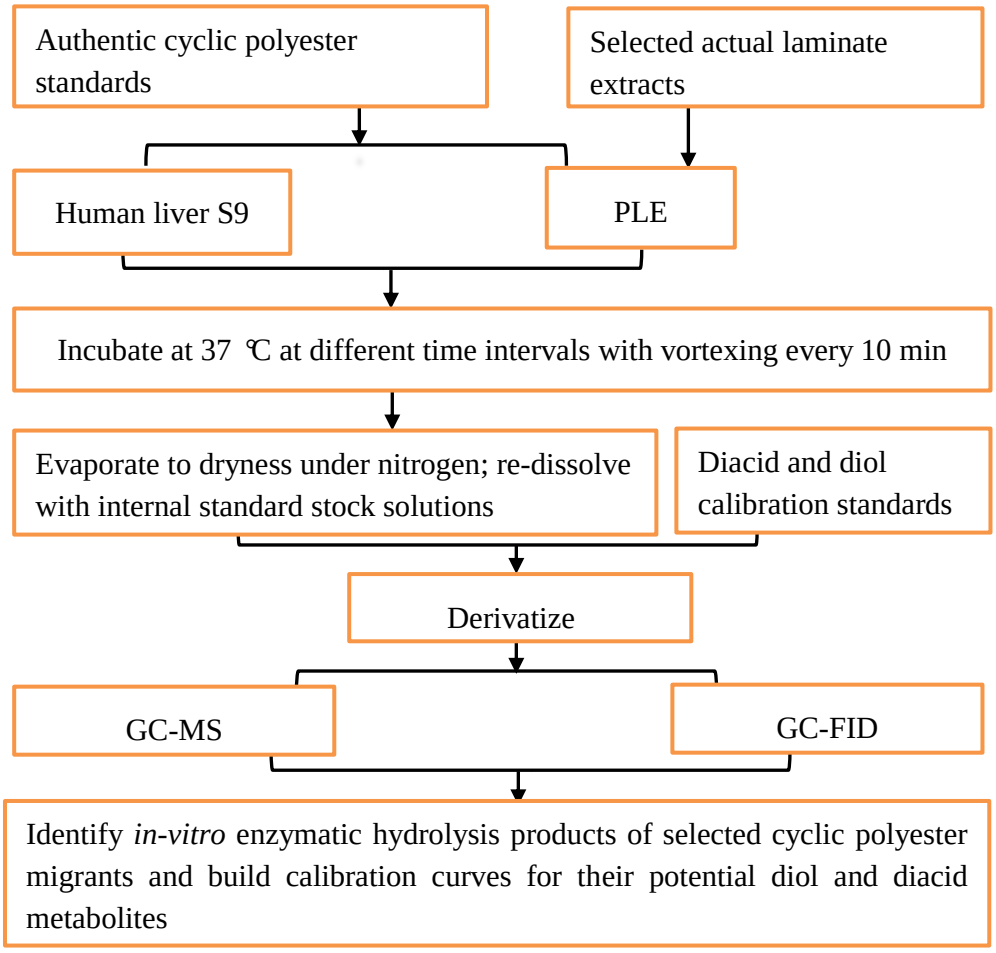


Figure 11: Flow Chart of Experimental Section 2

Section 3: Investigate the *in-vivo* metabolites of NPG-AA 2+2 which is potentially more hydrolysis resistant and toxic than the other selected ester migrants. In this section, we analyzed the plasma of the NPG-AA 2+2 treated mice by GC-MS-SIM. The experimental flow chart is shown in Figure 12.

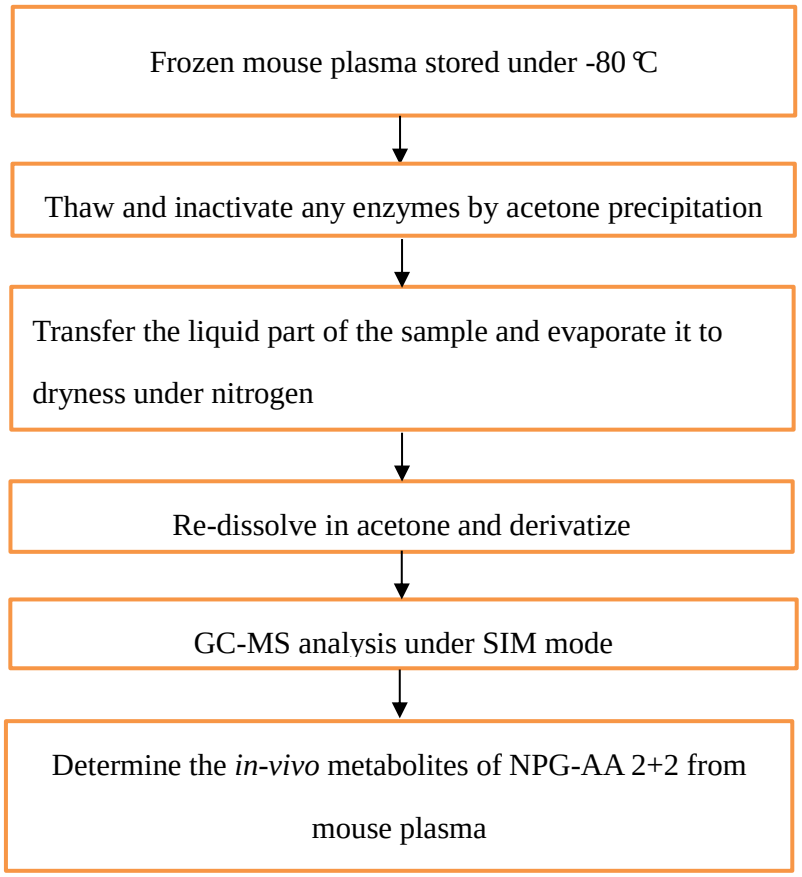


Figure 12: Flow Chart of Experimental Section 3

4.2 Reagents and Materials

Anthracene-d_{10} (CAS No. 1719-06-8, Lot 04021HO, 98% atom D) and Hexadecane (CAS No. 544-76-13 ReagentPlus, 99%) were purchased from Sigma Aldrich, Milwaukee, WI, USA.

Dichloromethane (Optima grade) and Acetone (HPLC grade) were purchased from Fischer Scientific, Fair Lawn, NJ, USA. Ethanol (USP grade) was purchased from Pharmco Products, Brookfield, CT, USA. Deionized water was prepared in house by Waters Milli-Q Nanopure ™ system.

Diethylene glycol (CAS No. 111-46-6, 99, 5%), adipic acid (CAS No. 124-04-9, 99%), isophthalic acid (CAS No. 121-91-5, 99.5%) and neopentyl glycol (CAS No. 126-30-7, 99%) were purchased from Sigma Aldrich, Saint Louis, MO, USA.

Derivatization reagent Sylon BFT (BSTFA + TMCS 99:1, 0.1ml /ampules) and pyridine (CAS 110-86-1, anhydrous, 99.8%) were purchased from Supelco Analytical, Bellefonte, PA, USA.

Synthesized authentic DEG-AA，DEG-IPA and NPG-AA 2+2 cyclic polyester standards and industrial laminates bonded with different kinds of polyurethane adhesives were provided by Coim, LLC.

Surfactant Triton™ X-100 (electrophoresis grade) (CAS 9002-93-1) was purchased from Fischer Scientific, Fair Lawn, NJ, USA.

Boric acid (CAS 10043-35-3) and sodium hydroxide (NaOH) (CAS 1310-73-2) were purchased from Fischer Scientific, Fair Lawn, NJ, USA.

Porcine liver esterase (PLE) (20units/mg CAS No. 9016-18-6) was ordered from Sigma Aldrich, Saint Louis, MO, USA.

Monopotassium phosphate (KH_2PO_4) (CAS 7778-77-0), potassium phosphate dibasic trihydrate ($K_2HPO_4 \cdot 3H_2O$) (CAS 16788-57-1) and magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$) (CAS 7791-18-6) were purchased from Sigma Aldrich Chemical, Saint Louis, MO, USA.

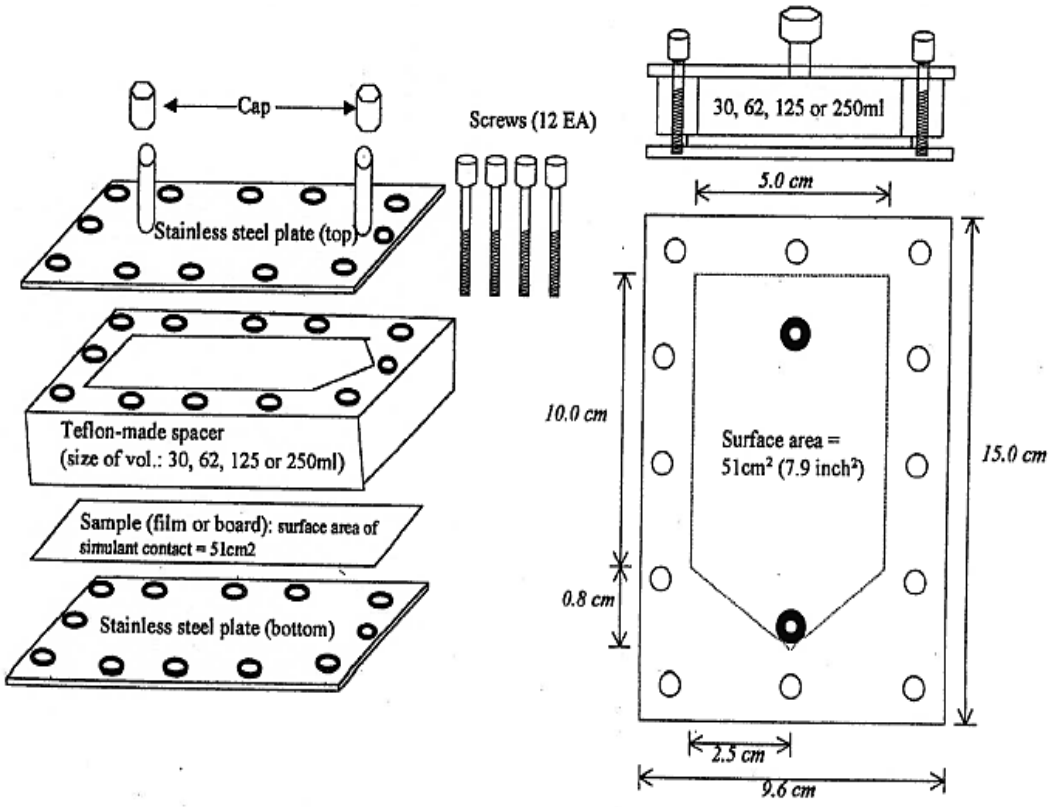
Human S9 liver protein (20 mg protein/ml, Product No.S2442), Glucose-6-phosphate dehydrogenase (G-6-P-DH) (100 Units/mg, CAS 9001-40-5), Glucose-6-phosphate (G-6-P) (CAS 56-73-5) and β-nicotinamide adenine dinucleotide phosphate hydrate (NADP) (CAS 53-59-8) were purchased from Sigma Aldrich, Saint Louis, MO, USA.

Plasma samples from mice dosed with different amounts of NPG-AA 2+2 were received from BioReliances lab under Coim Sponsorship. (Table 6)

Single side extraction cell (designed according to FDA specification for the food contact polymer migration by Dr. Thomas G. Hartman) is shown Figure 13. It consists of two stainless steel plates which sandwich a hollowed out Teflon® Spacer. The spacer isolates 51 cm^2 (7.9 $inch^2$) contact surface area (sealant side) and depending on the thickness of the spacer, each cell can hold 30, 62, 125 or 250 ml of food simulant to meet the specific volume-to-surface ratio requirements under the FDA conditions of use.

Table 6: Mouse Plasma Samples Information

Animal Number	Group/Sex	Body weight(g)	Volume(ml)	Dose Level(mg/kg)	Collection Time Point (min)
36	6/M	34.1	0.34	0	15
37	6/M	33.5	0.34	0	15
38	6/M	35.2	0.35	0	15
39	7A/M	35	0.35	500	15
40	7A/M	34.8	0.35	500	15
41	7A/M	33.4	0.33	500	15
42	8A/M	34.5	0.35	1000	15
43	8A/M	35.7	0.36	1000	15
44	8A/M	34.4	0.34	1000	15
45	9A/M	30.1	0.3	2000	15
46	9A/M	33.9	0.34	2000	15
47	9A/M	35	0.35	2000	15
48	7B/M	35.7	0.36	500	30
49	7B/M	32.8	0.33	500	30
50	7B/M	35.3	0.35	500	30
51	8B/M	35.6	0.36	1000	30
52	8B/M	36.4	0.36	1000	30
53	8B/M	32.5	0.33	1000	30
54	9B/M	35.7	0.36	2000	30
55	9B/M	37.5	0.38	2000	30
56	9B/M	37.4	0.37	2000	30



Figurer 13: Single-side Extraction Cell for Migration Test

[Designed by Dr. Thomas G. Hartman, Department of Food Science, Rutgers University]

4.3 Methods

4.3.1 Laminate Cell Extraction

In our investigation, each laminate film was cut and placed on the bottom steel plate with food contact surface area facing up with the Teflon spacer and top steel plate. The whole cell was then tightly assembled with 12 bolts. The food simulant 10% ETOH or 95% ETOH was introduced into the assembled extraction cell through the ports on the top plate. After capping the ports, the cells were incubated under FDA specified conditions according to the laminates' end uses in food packaging (Table 7).

Table 7: Food Types and Condition of Use for Migration Test

[Adapted from FDA, 2007]

Condition of Use	Description	Test Temperature and Time
A	High temperature, heat sterilized or retorted (ca. 121 ℃ (250 ℉)).	121 ℃ for 2Hrs then 40 ℃ for 10 Days
B	Boiling water sterilized.	100 ℃ for 2Hrs then 40 ℃ for 10 Days
C	Hot filled or pasteurized > 66 ℃ (150 ℉)	100 ℃ for 30 min (or 66 ℃ for 2Hrs) then 40 ℃ for 10 Days
D	Hot filled or pasteurized < 66 ℃ (150 ℉)	66 ℃ for 30 min then 40 ℃ for 10 Days
E	Room temperature filled and stored (no thermal treatment in the container)	40 ℃ for 10 Days
F	Refrigerated storage (no thermal treatment in the container)	20 ℃ for 10 Days
G	Frozen storage (no thermal treatment in the container)	20 ℃ for 5 Days
H	Frozen or refrigerated storage; ready prepared foods intended to be reheated in container at time of use	100 ℃ for 2Hrs
I	Irradiation (ionizing radiation)	No specifications, consult FDA
J	Cooking at temperatures exceeding 121 ℃ (250 ℉)	Perform test at the maximum intended cooking temperature and for the longest cooking time.

4.3.2 Extract Work Up

For a 10% ETOH simulant, the extract was directly transferred to a 50 ml bosilicate glass test tube, spiked with 100 ppb (weight to volume ratio) Anthracene-d_{10} internal standard, and vigorously extracted with 5.0 ml of dichloromethane (DCM). The tube was centrifuged at 2500 rpm for 30 min to promote complete phase separation, then the DCM

extract was transferred to a 5.0 ml tapered glass Reacti vial and concentrated under a gentle stream of nitrogen. Finally, 1 µl of the concentrated sample was injected into the GC-MS.

For a 95% ETOH extract, the procedure was similar except that only a 10 ml aliquot of the extract was transferred to a test tube followed by dilution with 35ml of distilled water. This is because 95% ETOH is miscible with DCM. The diluted extract was then processed as described above for GC-MS analysis.

4.3.3 Preparation of Authentic Cyclic Polyester Standards

Authentic standards of DEG-IPA, DEG-AA and NPG AA 2+2 in DCM were prepared in the concentrations of 10 mg/ml and diluted to 0.1 mg/ml respectively. The diluted standards were individually analyzed by GC-MS and GC-FID for purity, retention time and mass spectrum.

The DEG-AA standard contained DEG impurities that required purification by dissolving 1 g of DEG-AA in 10 ml DCM in 50 ml size bosilicate glass test tube and extracting with five 40 ml aliquots of deionized water. The remaining DCM extract was concentrated to dryness under a gentle stream of nitrogen, checked for purity by GC-MS and GC-FID, then prepared in concentrations of 10 mg/ml and 0.1 mg/ml as stock solutions.

4.3.4 PLE Enzyme Solution Preparation

pH 8.0 PLE enzyme buffer preparation: 0.01 M borate solution was prepared by dissolving 0.1545 g of boric acid in 250 ml deionized water at room temperature. The pH of the solution was adjusted to 8.0 with 0.01 M NaOH.

0.01% Triton X-100 enzyme buffer solution preparation: 1 ml of Triton X-100 was added to 9 ml of pH 8.0 buffer and further diluted 100 times with the pH 8.0 buffer.

PLE hydrolysis: 100μg of the synthesized authentic short chain cyclic polyesters or actual laminate cell extracts were dried under a gentle nitrogen stream in 5 ml Reacti vials. PLE solutions (20 units/ml or 200 units/ml) were freshly prepared by weighing 10 mg or 100 mg into 10 ml 0.01% Triton X-100 enzyme buffers. 100 μl of the PLE solution were added into each vial and the mixtures were incubated at 37 ℃ for different time intervals with vortexing every 10 min.

4.3.5 Human Liver S9 Fraction Hydrolysis

pH 7.4 potassium phosphate buffer preparation: A) 50mM KH_2PO_4-0.6805g KH_2PO_4 was dissolved in 100 ml deionized water. B) 50 mM K_2HPO_4-1.1411 g $K_2HPO_4 \cdot 3H_2O$ was dissolved in 100 ml deionized water. 19.2 ml of A were mixed with 80.2 ml of B and the pH was adjusted to 7.4.

NADPH generating system solution: 3.3 mM $MgCl_2$, 1.3mM NADP, 3.3mM G-6-P and 0.4 units/ml G-6-P-DH were prepared by dissolving 0.0335 g $MgCl_2 \cdot 6H_2O$, 0.0484 g NADP, 0.0429 g G-6-P and 0.2mg G-6-P-DH into 50 ml of the pH 7.4 potassium phosphate buffer.

Human liver S9 fraction hydrolysis: 100 μg of the synthesized authentic short chain cyclic polyesters were dried in 5 ml Reacti vials under a gentle stream of nitrogen. 10μl of the human liver S9 protein (20 mg/ml) and 90 μl of the NADPH generating system solution were added to the vial and mixed to disperse the polyesters. The reaction mixtures were incubated in the vials at 37 ℃ for an hour with vortexing every 10 min.

4.3.6 Calibration Curve Standard Solution

DEG, AA, IPA and NPG standards were prepared in a dynamic concentration range from 0.1-100 ppm with 50 ppm hexadecane internal standard acetone stock solutions.

4.3.7 Trimethylsilyl (TMS) Derivatization

The hypothesized hydrolysis products of cyclic polyesters have hydroxyl groups which will interact with the GC column and result in poor resolution. Therefore, TMS derivatization was used in our investigation to replace the active hydrogens, produce volatile analytes and prevent the undesired interactions.

Sylon BFT is a derivatization reagent containing BSTFA [N,O-bis (trimethylsilyl) trifluoroacetamide] and TMCS (trimethylchlorosilane) in the ratio of 99:1. The reaction mechanism involves nucleophilic attack upon silicon as shown in Figure 14.

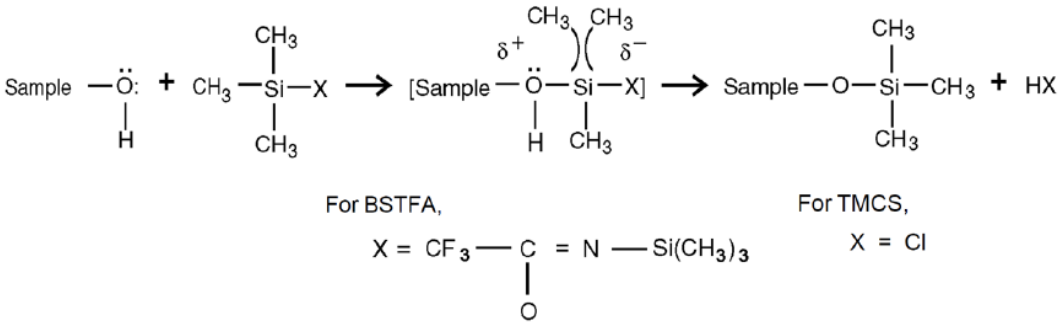


Figure 14: The Mechanism of Silylation. [Adapted from Knapp, 1979]

The experiment procedures are as follows: After incubation, the samples in reaction vials were evaporated to dryness under a gentle nitrogen stream. 1 ml of 50 ppm hexadecane in acetone internal standard stock solutions and 50 µl of Sylon BFT derivative reagents were added into each dried sample vial followed by heating and

periodically vortexing at 80 ℃ for 1 hour. 1 μl of this derivatized sample was directly injected in the GC-FID system.

For DEG, AA, IPA and NPG standards used for calibration curve building, the derivatization procedure was less complicated: 50 μl of the Sylon BFT reagent were spiked into 1 ml of the standard solution directly and heated at 80 ℃ for 1 hour.

4.3.8 Workup of Mouse Plasma Samples

The frozen mouse blood samples were stored at -80 ℃ and thawed at room temperature. Then 1.0 ml of acetone was added to each of the sample. After mixing well with a vortexor, the top liquid part of each sample was transferred to 2.0 ml size borosilicate glass autosampler vials that were sealed with Teflon-lined, screw cap closure. The samples were evaporated to dryness under nitrogen on a heating block set at 50 ℃. Immediately upon reaching dryness, 10.0 μl of pyridine (to stabilize the TMS derivatives), 50 μl of Sylon BFT derivatization reagent and 100 μl of acetone containing 1.0 μg of Anthracene-d_{10} internal standard were added. The samples were incubated for 60 minutes at 80 ℃ and then directly analyzed by GC-MS.

4.3.9 GC-MS Conditions

A Varian 3400 GC interfaced with a Finnigan TSQ-7000 triple stage quadrupole tandem mass spectrometer was used for all of the GC-MS analyses. The GC was installed with a 30 m×0.32 mm I.D×0.25 μm film thickness ZB-5MS (Phenomonex) capillary column. The injector temperature was set to 300 ℃ and column temperature was programmed from 50 ℃ (hold for 3 min) to 320 ℃ (hold for 5 min) at a rate of 10 ℃ per minute. Helium was used as carrier gas at 10 psi constant head pressure. Injection was in

splitless mode and septum purge was set to 100:1 split ratio which initiates at 0.5 min. The electron ionization mode (70 ev) was operated at 185 ℃ ion source temperature with a 35-750 scanning range. Data was all acquired and analyzed from the Xcaliber software.

For selected ion monitoring analysis of mouse blood samples, the GC-MS system was calibrated by analyzing freshly prepared standards of cyclic NPG-AA 2+2, derivatized NPG, AA and NPG-AA open ring monoester. The NPG-AA open ring monoester was produced via enzymatic hydrolysis of cyclic NPG-AA 2+2 by PLE. Based on the retention time and mass spectra of these compounds, a Selected Ion Monitoring table was constructed (Table 8).

Table 8: Selected Ion Monitoring Table for Mouse Plasma Metabolites Analysis

Compound	NPG-di-TMS	AA-di-TMS	Anthracene-d_{10}	NPG-AA open ring di-TMS	Cyclic NPG-AA 2+2
Time (min)	6-9	9-15	15-18	18-20	20-30
Selected ion (m/z)	143 158	111 275	188	111 291	215 428

4.3.10 GC-FID Conditions

GC-FID analyses were conducted on a Varian 3400 GC incorporated with a flame ionization detector. The column selection and temperature program was the same as the GC-MS analysis to make the results easy to compare. Helium was used as carrier gas at 10 psi. The detector temperature was set at 320 ℃, using air/hydrogen at a rate of 400/40

ml per minute, with helium as the make-up gas. Data was all acquired and analyzed from the Peak Simple software.

5 RESULTS & DISCUSSION

5.1 Identification of New Migrants by GC-MS

Twenty three new short chain cyclic polyesters were identified from our FDA single side cell migration tests on industry manufactured laminates. They are listed in Table 9 with their corresponding molecular ions and the relative abundance of ten major mass fragments. Additionally, we found 4 linear short chain oligoesters which are formed from two different diols and one diacid (Table 10).

The molecular weights of the identified cyclic polyesters ranged from 200 to 540 da and their fragmentation patterns were relatively complex. The basic fragmentation generally starts from the formation of an odd-electron ($OE^{+\bullet}$) molecular ion followed by McLafferty rearrangement with a C-O bond cleavage (McLafferty, 1993). Typical fragments were formed by the expulsion of H_2O, CO_2 and alkyl chains.

Fragment ions at m/z 55 ($C_4H_7{}^+$) and 111 ($C_6H_7O_2{}^+$) are characteristics for cyclic polyesters based on aliphatic acid (Table 9). For aromatic type esters, 149 ($C_8H_5O_3{}^+$) and 104 ($C_7H_4O^+$) were the common fragment ions, cyclic oligoesters that contain both aromatic and aliphatic acids exhibited mixed fragmentation characteristics. For example, NPG-AA-NPG-IPA showed major fragment ions at m/z 55, 104 and 149. Cyclic oligoesters from AA usually had a fragment ion at 129 ($C_6H_9O_3{}^+$). Cyclic oligoesters from SuA (suberic acid) generally had a fragment ion at 157 ($C_8H_{13}O_3{}^+$) because SuA has two more CH_2 in the alkyl chain than AA and their fragmentation pattern was very similar. AzA had three more CH_2 than AA in the alkyl chain, so its cyclic esters often

had a fragment ion at 171 ($C_9H_{15}O_3^+$). SeA had four more CH_2 than AA in the alkyl chain and its cyclic esters showed a characteristic fragment ion at 185 ($C_{10}H_{17}O_3^+$).

Most of the cyclic polyesters fragmented with their molecular ions intact. However, the molecular ion for NPG-AzA was not detectable because it was unstable under the high energy EI environment. In this case, Chemical Ionization (CI) analysis was required to verify the identification. The raw material component information of the industrial laminating adhesives also helped in confirming the identities of the short chain cyclic polyesters. Nevertheless, to elucidate identities of these compounds unequivocally, further analysis with synthesized authentic standards should be involved to confirm their retention times and mass spectra.

The newly identified ester migrants come from PU adhesives which are based on diols like 2-methyl-1, 3-propanediol (MP-diol), PG, 1, 6-HG and NPG and dicarboxylic acids like SeA, AzA, SuA, AA and IPA. MP-diol is a butylene glycol isomer and is mainly used for the synthesis of unsaturated polyesters. Because MP-diol reacts more quickly in esterification and polyesterification reactions that contribute to improved processability of the end products (Nalampang et al. 2003), it is gaining the attention of PU adhesive manufacturers. NPG is also a valuable raw material used in the production of PU adhesives. It has two methyl groups on the alpha carbon atom which contribute to the chemical and thermal stability of the NPG derived products. It is listed for food packaging adhesives in 21 CFR 175.105

Table 9: Newly Identified Cyclic Polyester Migrants from Laminates

Name	Molecular Ion (%)	10 Major Fragment Ion Peaks (%)			
MP-diol-AA	200 (0.56)	129(100.00)	111(83.88)	55(55.83)	101(29.99)
		41(26.17)	83(24.63)	172(22.46)	42(13.88)
		39(13.11)	112(12.80)		
MP-diol-AA 2+2	400 (11.56)	201(100.00)	127(18.51)	111(15.42)	400(11.56)
		329(10.41)	202(10.22)	341(8.85)	55(8.41)
		114(6.64)	129(6.13)		
1,6-HG-AA 2+2	456 (11.35)	207(100.00)	83(52.58)	229(51.32)	129(45.98)
		281(43.14)	55(43.01)	211(42.75)	111(33.56)
		82(28.95)	101(23.97)		
PG-AA 2+2	372 (35.22)	187(100.00)	113(66.62)	55(65.63)	111(60.65)
		100(41.60)	127(39.74)	372(35.22)	112(33.10)
		41(25.55)	126(25.50)		
NPG-AA-PG-AA	400 (11.48)	187(100.00)	55(60.53)	111(35.03)	113(25.08)
		215(19.48)	41(17.68)	69(16.64)	83(16.06)
		127(15.12)	101(14.73)		
NPG-AA-1,6HG-AA	442 (15.30)	55(100.00)	129(77.06)	215(60.62)	111(60.01)
		83(57.26)	141(45.01)	229(43.24)	82(30.57)
		101(27.57)	56(27.21)		
1,6HG-AA-PG-AA	414 (16.52)	55(100.00)	187(81.14)	113(67.09)	111(58.79)
		83(47.69)	129(41.64)	127(26.45)	100(26.03)
		101(25.65)	41(22.72)		
EG-SeA	228 (0.22)	185(100.00)	55(76.00)	98(72.72)	138(52.46)
		166(45.21)	41(44.93)	97(29.27)	86(25.12)
		99(24.20)	83(23.74)		
DEG-SeA	272 (0.23)	185(100.00)	55(36.08)	166(28.18)	41(27.26)
		69(23.12)	138(22.31)	56(21.20)	141(15.81)
		98(14.36)	187 (14.29)		
NPG-AA-NPG-IPA	448(7.29)	235(100.00)	149(44.59)	237(25.23)	69(24.10)
		104(17.10)	56(16.50)	363(15.56)	231(14.84)
		55(14.66)	41(14.08)		

Table 9: Newly Identified Cyclic Polyester Migrants from Laminates (Continued)

Name	Molecular Ion (%)	10 Major Fragment Ion Peaks (%)			
NPG-IPA 2+2	468(24.45)	384(100.00)	383(65.00)	209(40.10)	149(30.49)
		378(29.35)	281(26.20)	297(25.81)	468(24.45)
		208(24.17)	104(24.04)		
NPG-SeA	270 (1.66)	185(100.00)	55(49.27)	41(37.72)	98(31.08)
		141(23.51)	56(22.84)	166 (22.54)	138(22.04)
		69(20.97)	128(17.53)		
NPG-SeA 2+2	540 (26.37)	455(100.00)	456(54.69)	540(26.37)	141(15.40)
		450(13.77)	69(13.74)	271 (12.61)	185(11.64)
		128(10.78)	55(9.93)		
EG-SeA-DEG-SeA	498(70.23)	413(100.00)	498(70.23)	229(65.84)	55(52.15)
		455(51.05)	414(47.89)	69(47.39)	185(45.03)
		497(40.57)	141(36.38)		
DEG-AzA	258 (0.09)	55(100.00)	171(98.12)	152(82.61)	41(82.18)
		56(44.35)	69(36.75)	42 (35.46)	43(29.70)
		97(28.73)	83 (27.10)		
NPG-SuA	242(0.11)	157(100.00)	111(25.22)	175(23.44)	129(20.66)
		55(13.49)	41(11.76)	215(10.46)	56(9.37)
		158(8.52)	43(6.41)		
NPG-SuA 2+2	484(0.04)	215(100.00)	243(93.56)	429(77.29)	157(39.64)
		129(25.69)	389(19.95)	430(18.64)	371(16.84)
		111(16.46)	343(15.01)		
EG-PA 2+2	384(28.44)	341(100.00)	296(83.27)	44(58.01)	207(55.12)
		339(32.77)	384(28.44)	165(24.40)	324(23.63)
		355(19.96)	152(18.71)		
EG-TPA 2+2	384(0.65)	341(100.00)	338(30.67)	208(22.97)	342(19.91)
		104(8.24)	162(7.45)	324 (7.24)	236(6.54)
		266(6.20)	76(5.17)		
1,6HG-PA	248(1.35)	149(100.00)	59(8.83)	100(8.12)	82(7.84)
		54(5.61)	41(4.73)	67(4.42)	104(3.86)
		42(3.61)	71(3.00)		

Table 9: Newly Identified Cyclic Polyester Migrants from Laminates (Continued)

Name	Molecular Ion (%)	10 Major Fragment Ion Peaks (%)			
1,4BG-PA	238(0.28)	149(100.00)	59(14.62)	150(7.91)	205(5.27)
		41(5.10)	42(3.97)	43(3.80)	54(3.56)
		82(3.56)	104(3.28)		
EG-AzA	214(0.27)	152(100.00)	55(87.45)	41(52.12)	98(49.45)
		171(48.99)	83(30.76)	86(30.62)	84(26.78)
		124(24.98)	42(23.34)		
NPG-AzA	256(0)	55(100.00)	98(75.12)	41(67.41)	152(62.85)
		42(43.71)	171(39.66)	83(29.84)	43(27.55)
		84(27.38)	100(26.73)		

Table 10: Newly Identified Short Chain Polyester Migrants from Laminates

Name	Molecular Ion (%)	10 Largest Fragment Ion Peaks (%)			
DEG-PG-IPA	312(25.97)	237(100.00)	149(67.71)	45(37.35)	148(32.86)
		193(28.77)	147(27.80)	104(27.20)	312(25.97)
		309(25.46)	279(24.15)		
DEG-PG-AA	292(5.39)	217(100.00)	218(88.08)	173(75.35)	45(74.33)
		111(48.92)	155(39.74)	55(32.32)	44(29.35)
		99(24.93)	88(18.50)		
NPG-IPA-Butyl	308 (1.83)	149(100.00)	167(74.47)	235(55.34)	253(44.06)
		148(27.89)	166(23.49)	56(20.45)	233(19.26)
		251(18.70)	104(16.35)		
NPG-IPA-1,4 Butanediol	324 (18.50)	149(100.00)	235(92.91)	234(66.10)	157(52.06)
		71(45.57)	306(32.47)	43(25.61)	305(18.63)
		324(18.50)	323(16.35)		

5.2 Evaluation of the Occurrence of the Cyclic Polyester Migrants

From the migration testing data reports of 518 industry laminates for food packaging applications, we tabulated the frequency of occurrence of all cyclic polyester migrants with their individual maximum migration levels from the laminates (Table 11).

We found that DEG-AA was the most prevalent short chain cyclic polyester migrant with the relative frequency of occurrence at 0.6506 and its maximum migration level was 1593.05 ng/cm². DEG-AA 2+2 was the most prevalent dimer type cyclic ester migrant with the relative frequency of occurrence at 0.2278 and its maximum migration level was 1168.48 ng/cm². DEG-IPA was the most prevalent aromatic lactone migrant with the relative frequency of occurrence at 0.1293 and its maximum migration level was 36839.91 ng/cm². These three migrants were all based on DEG, the raw material commonly used in PU adhesive industry because of its relative cheap price and ether group functionality.

Since NPG offers superior performance for PU adhesives due to its high chemical and thermal stability, NPG based cyclic polyester migrants are frequently found in laminates. NPG-AA was the second most prevalent lactone migrant with the relative frequency of occurrence at 0.3263 and its maximum migration level was 1495.57 ng/cm². NPG-AA 2+2 was the second most prevalent cyclic polyester dimer migrant with relative frequency of occurrence at 0.1776 and its maximum migration level was 2248.64 ng/cm².

Table 11: Occurrence of Cyclic Polyester Migrants in Laminate Packagings

Compound	Maximum Migrant Concentration (ng/cm2)	Frequency of Occurrence	Relative Frequency of Occurrence
DEG-AA	1593.05	337	0.6506
NPG-AA	1495.57	169	0.3263
1,6 HG-AA	307.98	129	0.2490
DEG-AA 2+2	1168.48	118	0.2278
1,4 BG-AA	115.17	108	0.2085
NPG-AA 2+2	2248.64	92	0.1776
EG-AA	149.48	90	0.1737
DEG-IPA	36839.91	67	0.1293
1,4 BG-AA 2+2	74.99	45	0.0869
1,6 HG-AA2+2	134.78	24	0.0463
DEG-PA	1250.05	15	0.0290
DEG-AzA	17.89	14	0.0270
EG-AA 2+2	68.15	14	0.0270
EG-AzA	27.89	13	0.0251
NPG-AzA	135.28	13	0.0251
DEG-SeA	161.52	12	0.0232
EG-IPA 2+2	711.79	9	0.0174
DEG-IPA 2+2	745.83	9	0.0174
DEG-AA-EG-AA	126.95	9	0.0174
NPG-AA-NPG-IPA	257.43	7	0.0135
EG-SeA	175.54	7	0.0135
EG-SeA-DEG-SeA	246.91	6	0.0116
NPG-SeA 2+2	294.15	6	0.0116

Table 11: Cyclic Polyester Migrants' Occurrence in Laminate Packagings (Continued)

Compound	Maximum Migrant Concentration (ng/cm2)	Frequency of Occurrence	Relative Frequency of Occurrence
NPG-SeA	196.16	6	0.0116
NPG-IPA 2+2	48.39	5	0.0097
DEG-PA 2+2	5.45	4	0.0077
DEG-AA-EG-IPA	529.57	3	0.0058
NPG-SuA 2+2	129.18	3	0.0058
NPG-SuA	6.32	2	0.0039
EG-IPA-EG-AA	230.82	2	0.0039
DEG-IPA-DEG-AA	408.39	2	0.0039
DEG-IPA-EG-IPA	1064.48	2	0.0039
1,4 BG-PA	2.59	1	0.0019
1,6 HG-PA	607.06	1	0.0019
MP Diol-AA	28.41	1	0.0019
MP-Diol AA 2+2	91.08	1	0.0019
EG-PA 2+2	781.51	1	0.0019
2EG-2TPA	40.82	1	0.0019
PG-AA 2+2	5.58	1	0.0019
NPG-AA-PG-AA	11.81	1	0.0019
1,6HG-AA-PG-AA	19.71	1	0.0019
NPG-AA-1,6HG-AA	16.17	1	0.0019

Usually the presence of the monomeric type cyclic polyester migrants such as DEG-AA and NPG-AA are accompanied by their associated dimer type polyesters such as DEG-AA 2+2 and NPG-AA 2+2 because the formation of these cyclic polyester

by-products during polyester polyol manufacture is unavoidable and beyond our control. Also，laminate manufacturers usually blend different polyester polyol raw materials together in order to obtain desired adhesion properties, so several different cyclic polyesters and their combinations are usually found together from the same laminate. Figure 15 shows the GC-MS chromatogram of a typical laminate sample extract in which EG-AA. DEG-AA, DEG-AA 2+2, NPG-AA, NPG-AA 2+2, 1,6 hexanediol-AA and 1,6 hexanediol-AA 2+2 were detected as migrants.

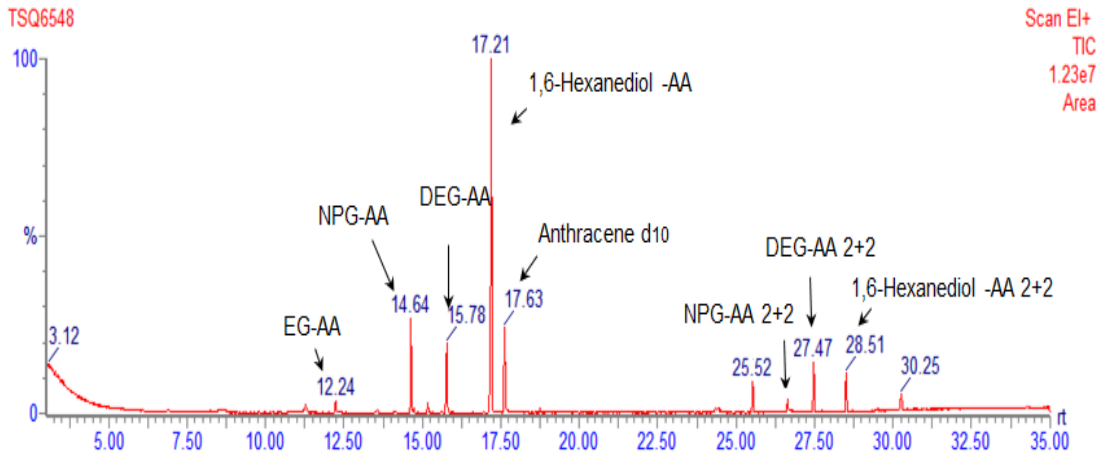


Figure 15: GC-MS Chromatogram of a Coffee Bean Laminate Packaging Extract

5.3 Cyclic Polyesters Hydrolysis by PLE

Based on the relative occurrence frequency of the cyclic polyester migrants (Table 11), we chose DEG-AA, DEG-IPA, NPG-AA and NPG-AA 2+2 as candidate compounds for our hydrolysis study. Figure 16 shows the GC-MS chromatogram of the three authentic standards - DEG-AA, DEG-IPA and NPG-AA 2+2 supplied by our sponsor. Their respective retention times are 15.82 min, 18.67 min and 26.68 min respectively.

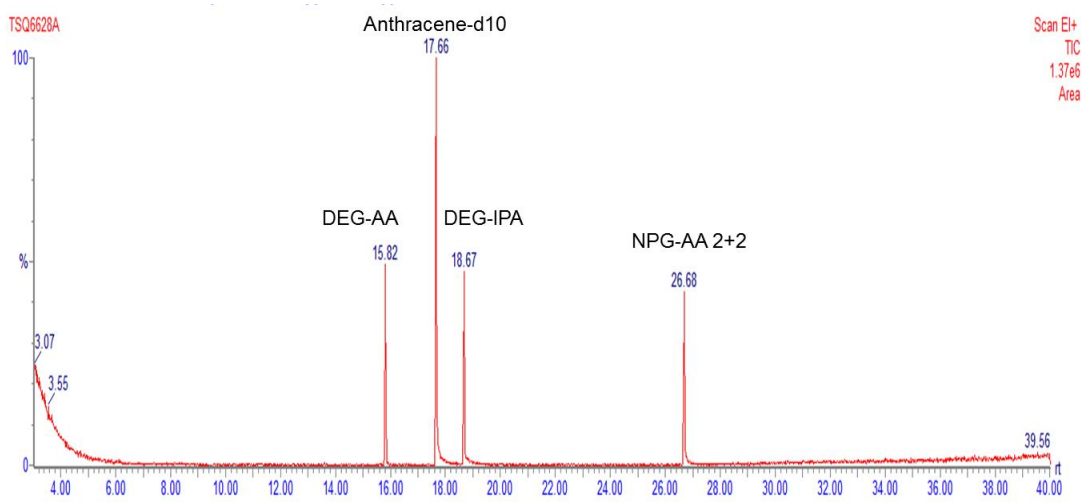


Figure 16: GC-MS Chromatogram of a Mixture of Sythesized DEG-AA, DEG-IPA and NPG-AA Synthesized Standards

5.3.1 Authentic Cyclic Polyester Standards Hydrolysis by PLE

Compared to the control without PLE treatment (Figure 17), 100μg of DEG-IPA authentic standard was completely decomposed to DEG, IPA and DEG-IPA monoester after 40 min incubation with 20 U of PLE at 37 ℃ (Figure 18). Unlike the aromatic type diester plasticizer DEHP which only breaks down to the monoester and 2-ethylhexynol in enzymatic hydrolysis (Niino et al., 2003), aromatic dicarboxylic acid showed up as the decomposition product of DEG-IPA. Figure 19 shows the mass spectrum of DEG-IPA monoester di-TMS derivative: the characteristic ions at m/z 383 resulted from loss of CH_3^+ from the molecular ion and the ion at 73 ($Si(CH_3)_3^+$) is from the trimethylsilyl fragment. Because the metabolites of the cyclic oligoesters all have more than one functional group site to react with the derivatization agents, additional TMS derivative

peaks or some derivatives' decomposition products generally showed in the enzyme treated hydrolyzate chromatogram (Figure 19).

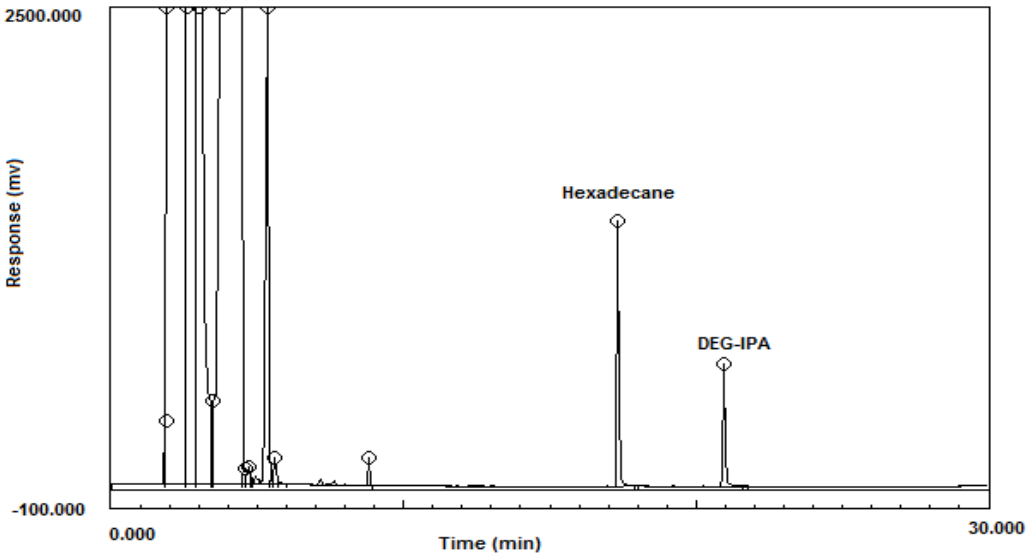


Figure 17: GC Chromatogram of DEG-IPA Control Hydrolyzate without PLE

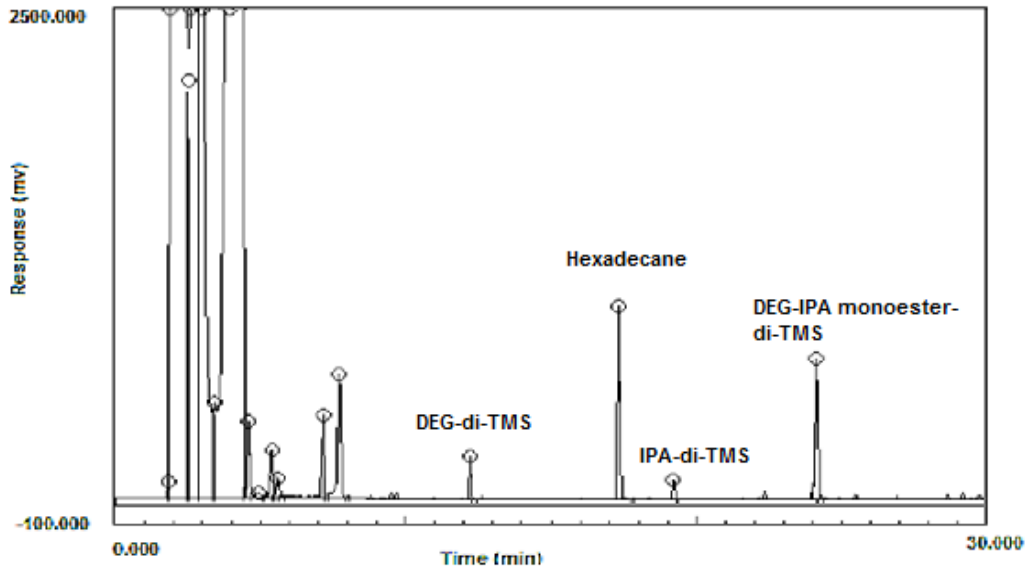


Figure 18: GC Chromatogram of DEG-IPA Hydrolyzate Obtained after Incubation with PLE (20U) at 37 ℃ for 40 minutes

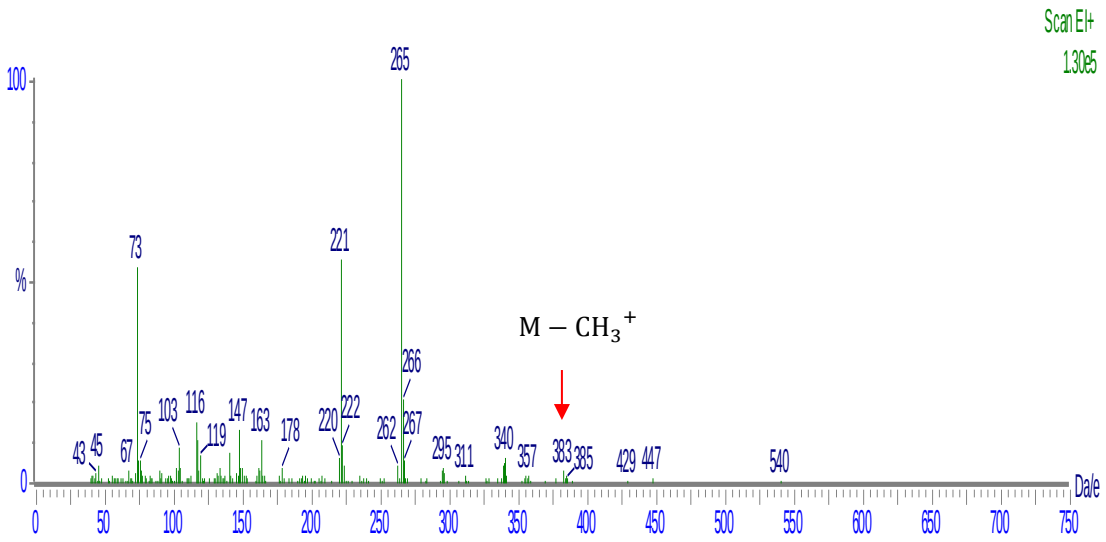


Figure 19: EI Mass Spectrum of DEG-IPA Monoester Di-TMS

According to Figures 20 and 21, the aliphatic type cyclic polyester DEG-AA was less resistant to hydrolysis than the aromatic type cyclic polyester DEG-IPA under the same test conditions. This is consistent with previous reviewed hydrolysis studies on aliphatic type and aromatic type polyester by Tokiwa et al. (1977) and Takahashi et al. (1981). In the absence of PLE enzyme, 100µg of DEG-AA authentic standard remains intact after 40 min incubation at 37 ℃ (Figure 20). In the presence of 20 U PLE, 100µg of DEG-AA authentic standard was completely decomposed to DEG and AA after 40 min incubation at 37 ℃ (Figure 21). DEG-AA monoester was not observed because it was already hydrolyzed to the monomers. After reducing PLE from 20 U to 2 U, DEG-AA monoester showed up as the hydrolysis products under the same incubation conditions as above (Figure 22). Figure 23 shows the mass spectrum of DEG-AA monoester di-TMS derivative: the characteristic ions at m/z 363 resulted from loss of CH_3^+ from the molecular ions

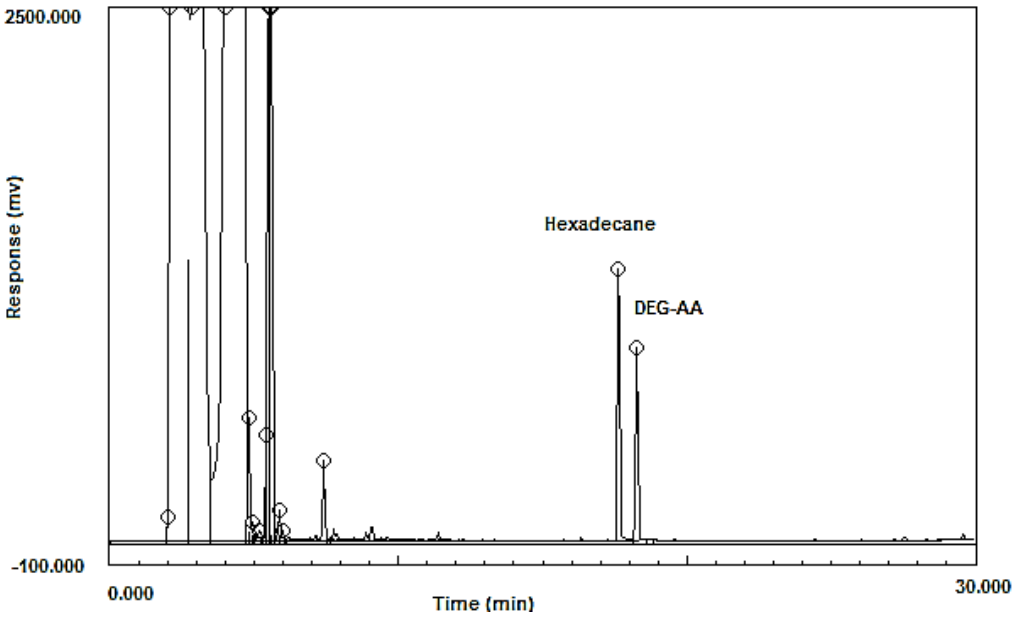


Figure 20: GC Chromatogram of DEG-AA Control Hydrolyzate without PLE

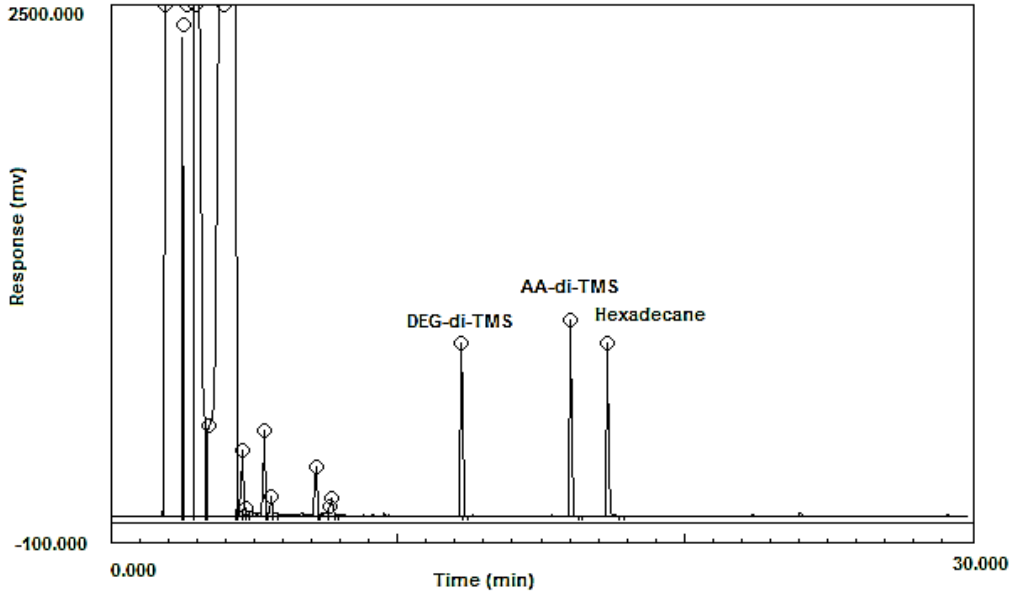


Figure 21: GC Chromatogram of DEG-AA Hydrolyzate Obtained after Incubation with PLE (20U) at 37 ℃ for 40 minutes

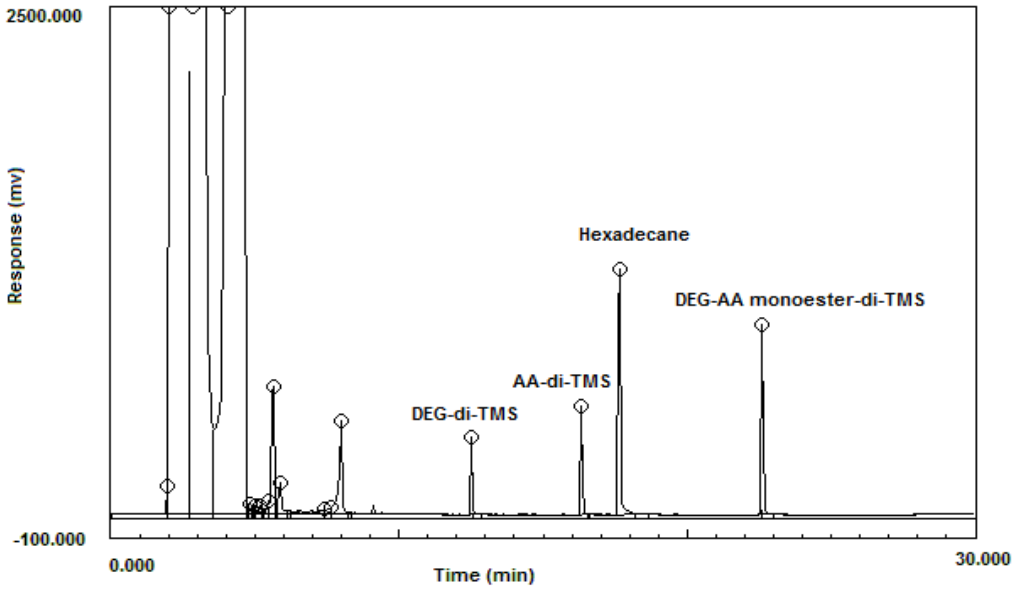


Figure 22: GC Chromatogram of DEG-AA Hydrolyzate Obtained after Incubation with PLE (2U) at 37 ℃ for 40 minutes

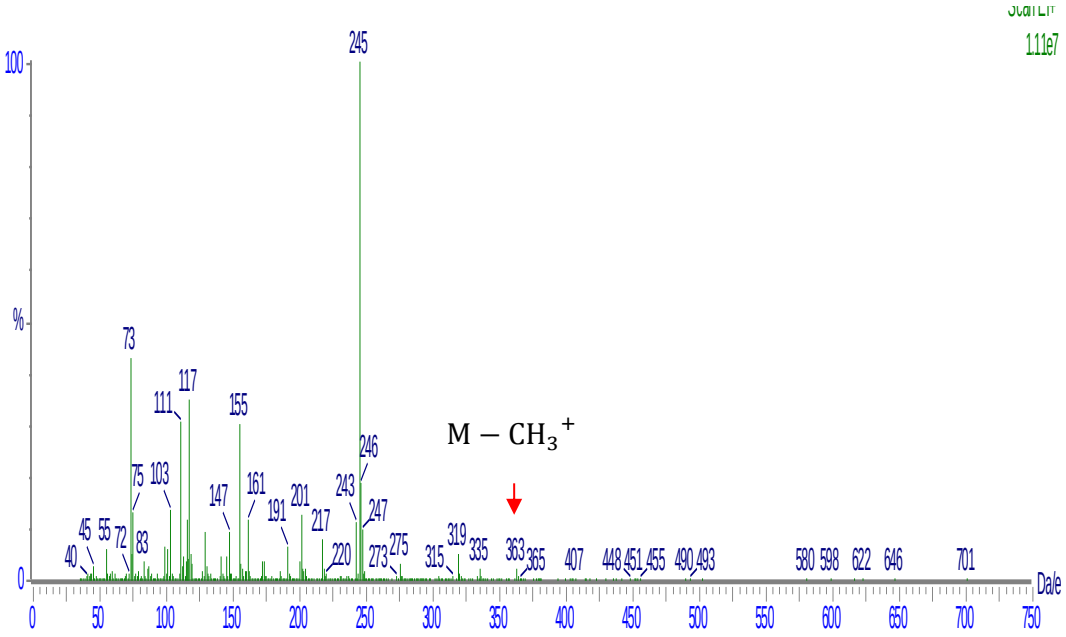


Figure 23: EI Mass Spectrum of DEG-AA Monoester di-TMS

Figures 24 and 25 show that the dimer type cyclic polyester NPG-AA 2+2 were even more resistant to hydrolysis than DEG-IPA under the same test conditions. Compared to the control without PLE treatment, NPG-AA 2+2 only partially decomposed to the NPG, AA and NPG-AA monoester after incubation with 20 units of PLE at 37 ℃ for 40 minutes (Figure 25). The open ring NPG-AA 2+2monoester was not observed in our hydrolysis, consistent with the cyclic PET-trimer hydrolysis results from Hooker et al (2003). The NPG-AA 2+2 monoester was expected to be already hydrolyzed because the enzymatic reaction is very fast. The mass spectrum of NPG-AA monoester di-TMS derivative with the molecular ion at 375 is shown in Figure 26.

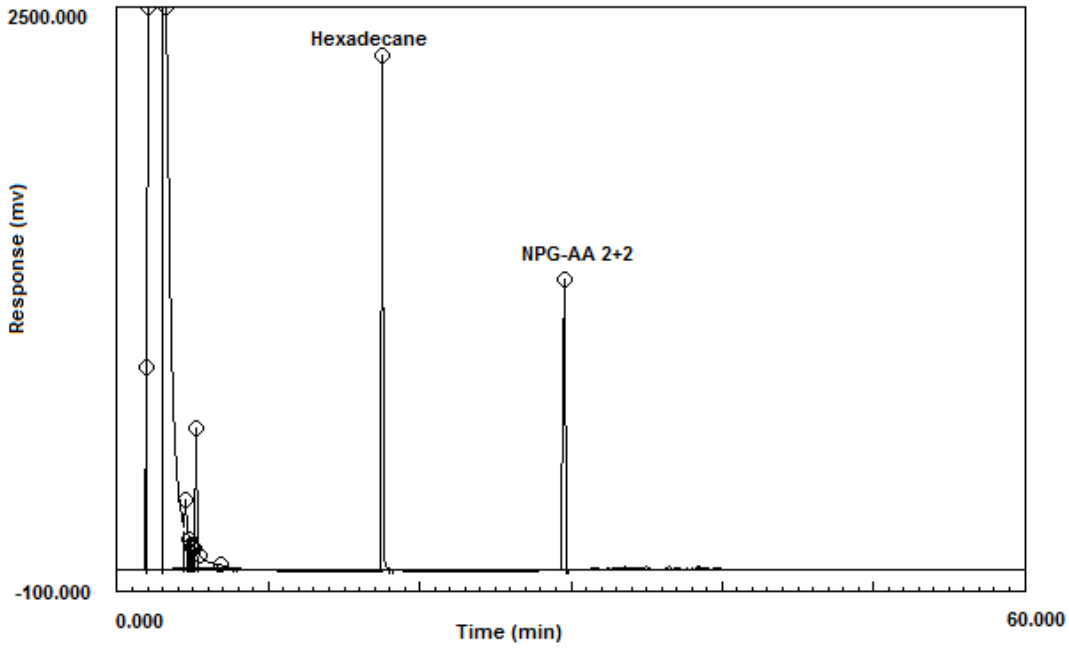


Figure 24: GC Chromatogram of NPG-AA 2+2 Control Hydrolyzate without PLE

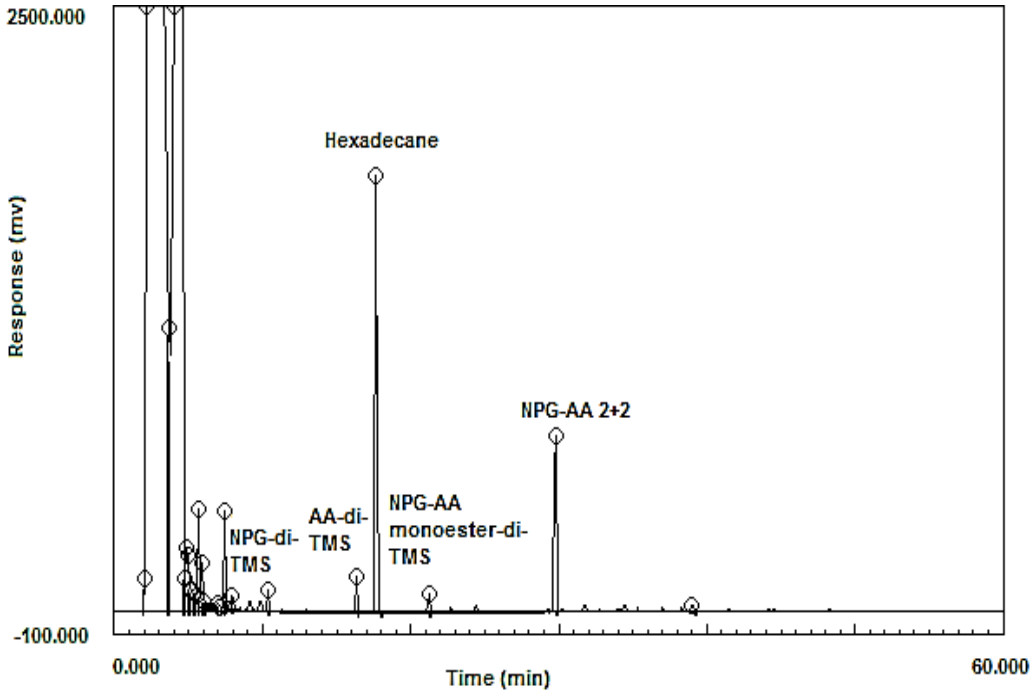


Figure 25: GC Chromatogram of NPG-AA 2+2 Hydrolyzate Obtained after Incubation with PLE (20U) at 37 ℃ for 40 minutes

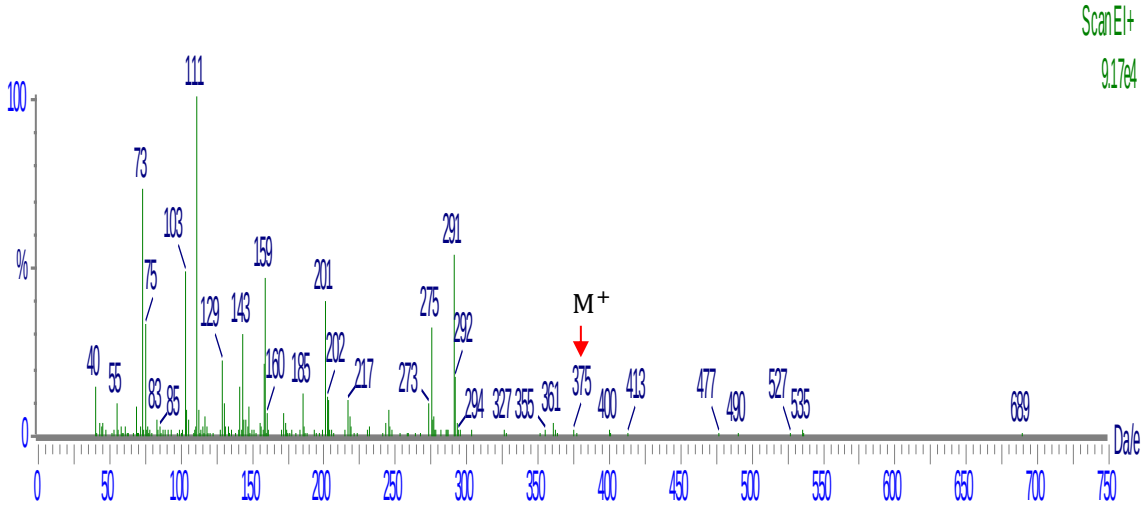


Figure 26: EI Mass Spectrum of NPG-AA Monoester di-TMS

The discovery of the intermediate open ring monoesters in our studies proves that short chain cyclic polyester migrant hydrolysis is a stepwise process with initial ring cleavage followed by a complete breakdown of the compounds to their corresponding

diols and dibasic acids. The resistance of cyclic oligoester migrants to PLE hydrolysis under the same conditions can be ranked from high to low as dimer type NPG-AA 2+2 > aromatic type DEG-IPA > aliphatic type DEG-AA.

5.3.2 Laminate Extracts Hydrolysis by PLE

Because there is no authentic standard available for NPG-AA, we selected the actual laminate extract which had only NPG-AA and NPG-AA 2+2 as cyclic polyester migrants (Figure 27) for the PLE hydrolysis study. Figure 28 shows that NPG-AA and NPG-AA 2+2 were completely decomposed to NPG and AA. The average amount of NPG-AA and the amount of NPG-AA 2+2 in the actual laminate extract were less than the authentic NPG-AA 2+2 standard for hydrolysis (Section 5.3.1). The authentic NPG-AA 2+2 standard substrate for previous PLE hydrolysis was 100 μg, while the NPG-AA and NPG-AA 2+2 extracted from the laminate was approximately 4.5942 μg and 32.232 μg based on the internal standard area ratio estimation from our data reports. Consequently, the enzyme to substrate ratio was higher in this experiment and presumably accounted for complete breakdown of NPG-AA and NPG-AA 2+2.

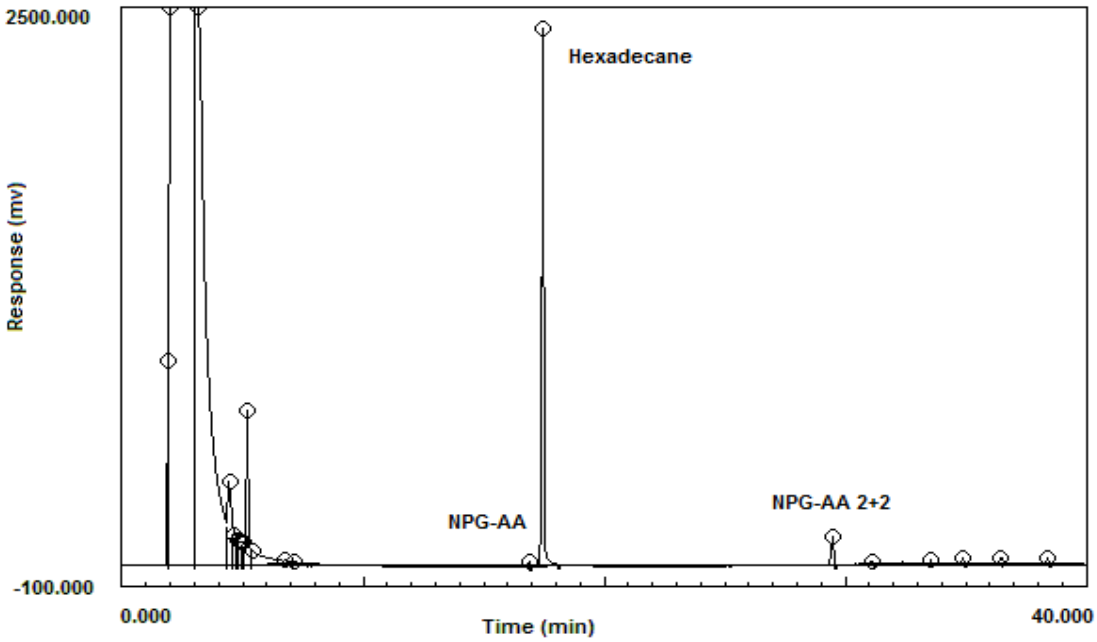


Figure 27: GC Chromatogram of NPG-AA and NPG-AA 2+2 (from Laminate Extract) Control without PLE

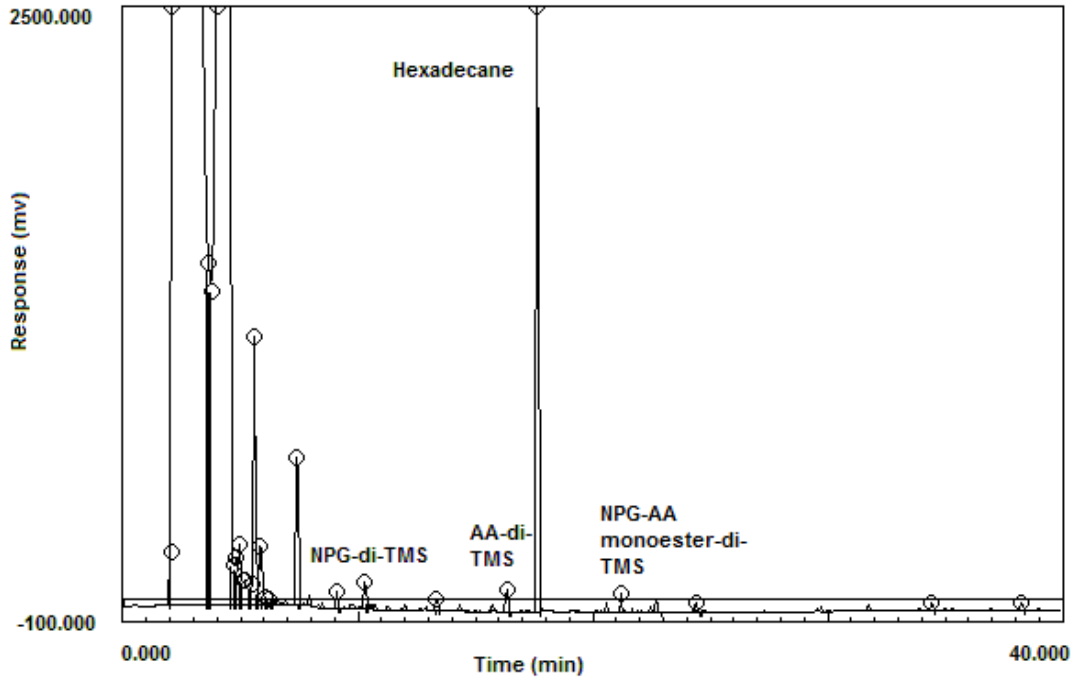


Figure 28: GC Chromatogram of NPG-AA and NPG-AA 2+2 (from Laminate Extract) Hydrolyzate Obtained after Incubation with PLE (20U) at 37 ℃ for 40 minutes

Since MP-diol based PU adhesives are relative new and have great market potential, we also selected a real laminate extract which had only MP-diol-AA and MP-diol-AA 2+2 as cyclic polyester migrants (Figure 29) for PLE hydrolysis. Figure 30 shows that MP-diol-AA and MP-diol-AA 2+2 were completely hydrolyzed to MP-diol and AA. The average amount of MP-diol-AA and MP-diol-AA 2+2 migrants extracted from the selected laminate were approximately 1.45 μg and 4.645 μg respectively. Their monoester intermediates may have already been decomposed, consistent with Shrikhande's preliminary hydrolysis studies on cyclic oligoester migrants from laminate extract.

Therefore, cyclic oligoester migrants NPG-AA and NPG-AA 2+2, MP-diol and MP-diol 2+2 from laminate can be completely broken down by PLE hydrolysis.

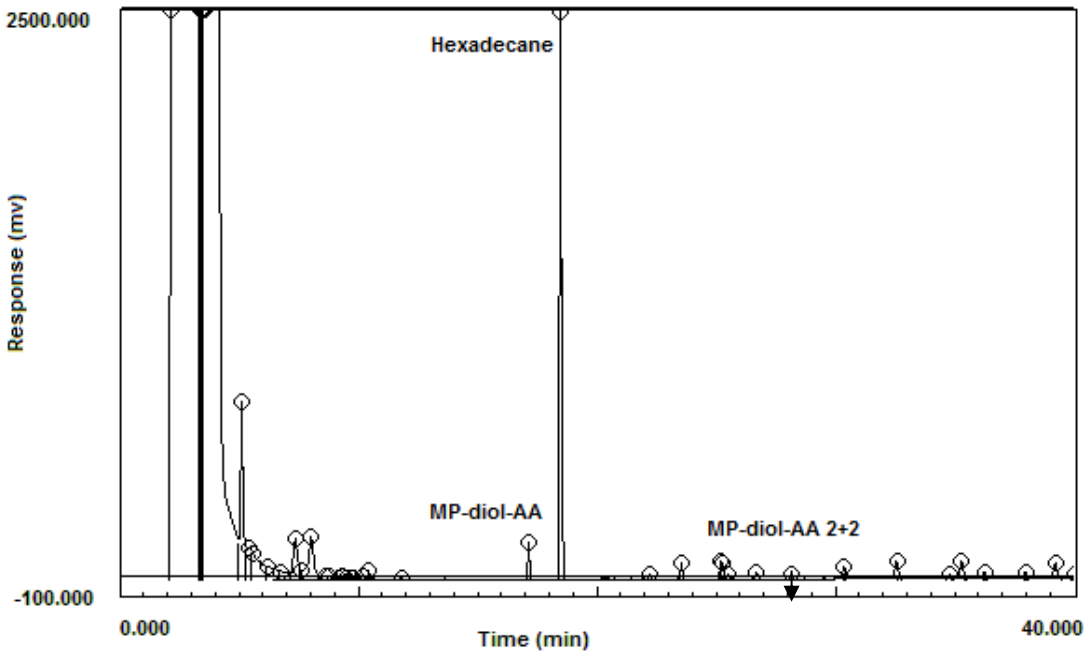


Figure 29: GC Chromatogram of MP-diol-AA and MP-diol-AA 2+2 (from Laminate Extract) Control without PLE

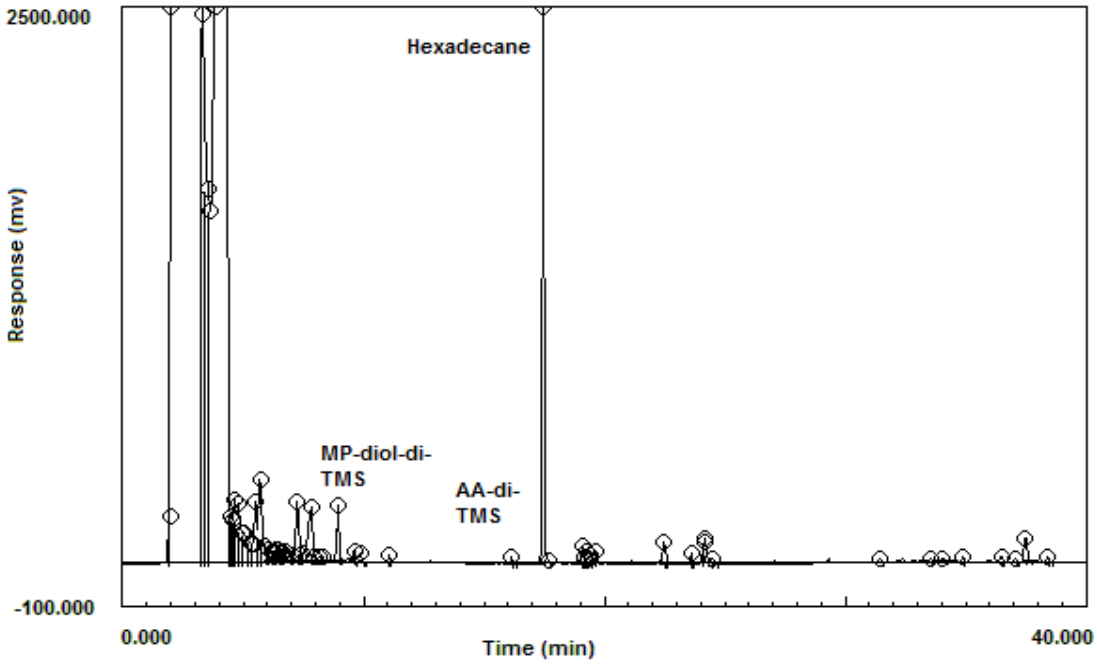


Figure 30: GC Chromatogram of MP-diol-AA and MP-diol-AA 2+2 (from Laminate Extract) Hydrolyzate Obtained after Incubation with PLE (20U) at 37 ℃ for 40 minutes

5.3.3 The Effects of Experiment Parameters on PLE Hydrolysis

We also performed preliminary experiments to explore effects of incubation time, enzyme concentration and addition of surfactant on hydrolysis of authentic DEG-AA, DEG-IPA and NPG-AA 2+2 standards by PLE (Table 12).

To determine the effect of time on the percent hydrolysis of cyclic oligoesters, we terminated the hydrolysis at certain time intervals. With 20 U of PLE and 0.01% Triton X-100, the percent hydrolysis of NPG-AA 2+2 increased continually and the percent intermediate of NPG-AA monoester decreased continually with the increasing incubation time. After 20 min incubation, only 8.9% NPG-AA 2+2 was hydrolyzed. After 24 h incubation, 68.86% of NPG-AA 2+2 was hydrolyzed. For DEG-IPA and DEG-AA, the substrates were completely hydrolyzed by 20 U of PLE with 0.01% Triton X-100 after 20

min incubation. The percent DEG-IPA monoester decreased from 32.29% at 20 min to 27.91% at 60 min and the percent DEG-AA monoester decreased from 2.53% to 1.80% at the same time intervals. Hence, hydrolysis reactions of DEG-IPA and DEG-AA were very fast and shorter incubation intervals between 0 to 20 min should be used to monitor the hydrolysis events.

The effect of surfactant on DEG-AA hydrolysis was evaluated by withholding 0.01% Triton X-100. Without Triton X-100, DEG-AA was still completely hydrolyzed after 60 min incubation, but the percent DEG-AA monoester increased to 5.01% because Triton X-100 is a nonionic surfactant which can increase the reaction surface area and reduce the agglomerates formation (Hooker et al., 2003). This is especially important for larger molecule cyclic polyester dimers and trimers.

After reducing 20 U of PLE to 2U and in the absence of Triton X-100, the percent hydrolysis of DEG-AA was 94.17% at 20 min, 98.89% at 40min and 98.05% at 60min of incubation. Concurrently, the percent of DEG-AA monoester decreased from 61.13% at 20 min to 46.8% at 40 min and 33.00% at 60 min. Therefore, higher enzyme concentration contributes to more complete hydrolysis of cyclic oligoester migrants.

Table 12: Cyclic Polyester Hydrolysis Dependence Factors

Experiment	Substrate	Incubation Time (min)	Triton X-100 (%)	Enzyme (U)	Percent Cyclic Oligoester Hydrolysis (%)	Percent Open Ring Monoester (%)
Time Dependence	DEG-AA	0	0.01	20	0.00	—
		20	0.01	20	100.00	2.53
		40	0.01	20	100.00	2.09
		60	0.01	20	100.00	1.80
	DEG-IPA	0	0.01	20	0.00	—
		20	0.01	20	100.00	32.29
		40	0.01	20	100.00	30.14
		60	0.01	20	100.00	27.91
	NPG-AA 2+2	0	0.01	20	0.00	—
		20	0.01	20	8.90	9.43
		40	0.01	20	18.52	4.78
		60	0.01	20	10.41	3.70
		120	0.01	20	26.29	1.00
		240	0.01	20	50.03	0.47
		1440	0.01	20	68.86	0.20
Surfactant Dependence	DEG-AA	60	—	20	100.00	5.01
Enzyme Concentration Dependence	DEG-AA	20	—	2	94.17	61.13
		40	—	2	98.89	46.80
		60	—	2	98.05	33.00

5.4 Calibration Curves

According to the Hazardous Substances Data Bank (HSDB) from the National Library of Medicine (NLM), AA has very low toxicity in rats, with oral LD50 > 11000 mg/kg. IPA also exhibits low toxicity with oral LD50 > 5000 mg/kg (no deaths) to 13000 mg/kg in rats. DEG has moderate acute toxicity in animal experiments and the LD50 = 12565 mg/kg in rats. However, some research suggests that the lethal dose in adults is 1 mL/kg because it appears more hazardous to humans than implied by oral toxicity data in laboratory animals (Schep, 2009). NPG acute toxicity is considered as moderate with oral LD 50 = 3200 mg/kg in rat.

Since there is abundant toxicity information available for the diacids and diols from DEG-AA, NPG-AA, DEG-IPA and NPG-AA 2+2 hydrolysis, we constructed calibration curves to quantify these monomers (Figures 31 to 34). The calibration curves were plotted by the peak area ratio of derivatized AA, IPA, NPG and DEG to internal standard versus the concentrations of standard solutions of AA, IPA, NPG and DEG. For AA, NPG and DEG, a seven point calibration was performed (Tables 13 to 16). The dynamic range of calibration was approximately 0.1 ppm to 50 ppm (0.01~5 μg/100 μl). For IPA, an eight point calibration was performed (Table 16). The dynamic range of calibration is approximately 0.1ppm to 100ppm (0.01~10 μg/100μl). All of the calibration curves were linear in their dynamic range (R-squared > 0.99). Higher concentrations of AA, NPG, DEG and IPA tended to be non-linear. Since the TMS derivatives are very susceptible to hydrolysis and decomposition during GC-FID analysis, all of the calibration standards must be freshly and carefully prepared.

Table 13: GC-FID AA Calibration Data

AA Conc.in µg/ml w/v	Hexadecane Int. Std. Conc. in µg/ml w/v	Peak Area Ratio AA/Int. Std.	AA Regression Output:	
0.101	50	0.00009	Constant	-0.017828
0.505	50	0.00022	Std Err of Y Est	0.009432
1.01	50	0.00224	R Squared	0.992387
5.05	50	0.02001	No. of Observations	7
10.1	50	0.08401	Degrees of Freedom	5
25.25	50	0.23406	X Coefficient(s)	0.0110628
50.5	50	0.55645	Std Err of Coef.	0.000433

Table 14: GC-FID NPG Calibration Data

NPG Conc.in µg/ml w/v	Hexadecane Int. Std. Conc. in µg/ml w/v	Peak Area Ratio NPG/Int. Std.	NPG Regression Output:	
0.102	50	0.01466	Constant	0.034449
0.51	50	0.02426	Std Err of Y Est	0.010412
1.02	50	0.04273	R Squared	0.992212
5.1	50	0.10206	No. of Observations	7
10.2	50	0.19197	Degrees of Freedom	5
25.5	50	0.35225	X Coefficient(s)	0.011955
51	50	0.63017	Std Err of Coef.	0.000474

Table 15: GC-FID DEG Calibration Data

DEG Conc.in µg/ml w/v	**Hexadecane Int. Std. Conc. in µg/ml w/v**	**Peak Area Ratio DEG/Int. Std.**	**DEG Regression Output:**	
0.10098	50	0.00018	Constant	0.003257
0.5049	50	0.00034	Std Err of Y Est	0.007554
1.0098	50	0.01780	R Squared	0.993990
5.049	50	0.06699	No. of Observations	7
10.098	50	0.11563	Degrees of Freedom	5
25.245	50	0.22790	X Coefficient(s)	0.009982
50.49	50	0.51730	Std Err of Coef.	0.000347

Table 16: GC-FID IPA Calibration Data

IPA Conc.in µg/ml w/v	**Hexadecane Int. Std. Conc. in µg/ml w/v**	**Peak Area Ratio IPA/Int. Std.**	**IPA Regression Output:**	
0.1	50	0.00419	Constant	-0.0086
0.5	50	0.00835	Std Err of Y Est	0.009034
1	50	0.02082	R Squared	0.999220
5	50	0.07544	No. of Observations	7
10	50	0.19947	Degrees of Freedom	5
25	50	0.43521	X Coefficient(s)	0.019460
50	50	0.97363	Std Err of Coef.	0.000222
100	50	1.94264102		

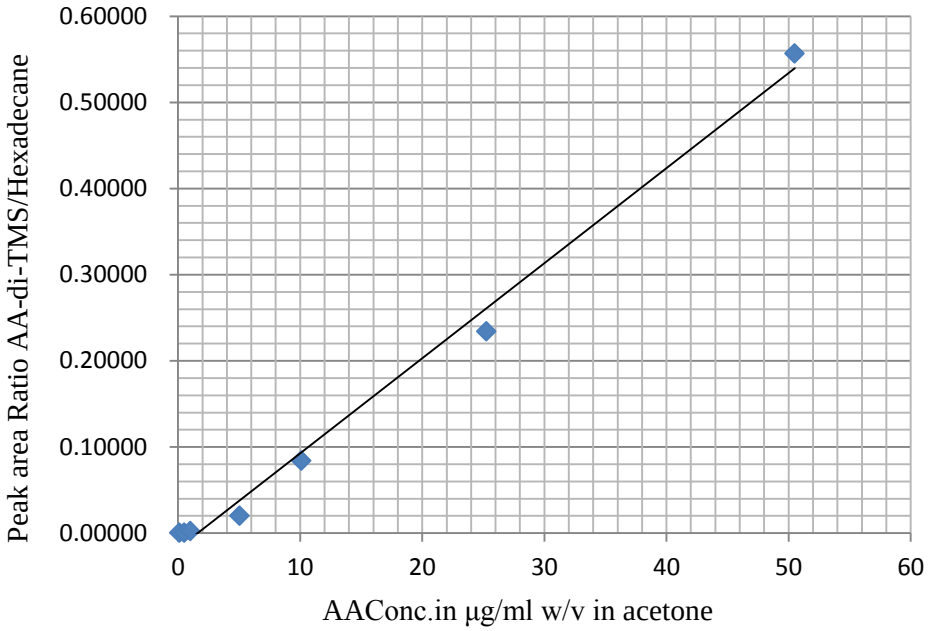


Figure 31: GC-FID AA-di-TMS Calibration Curve

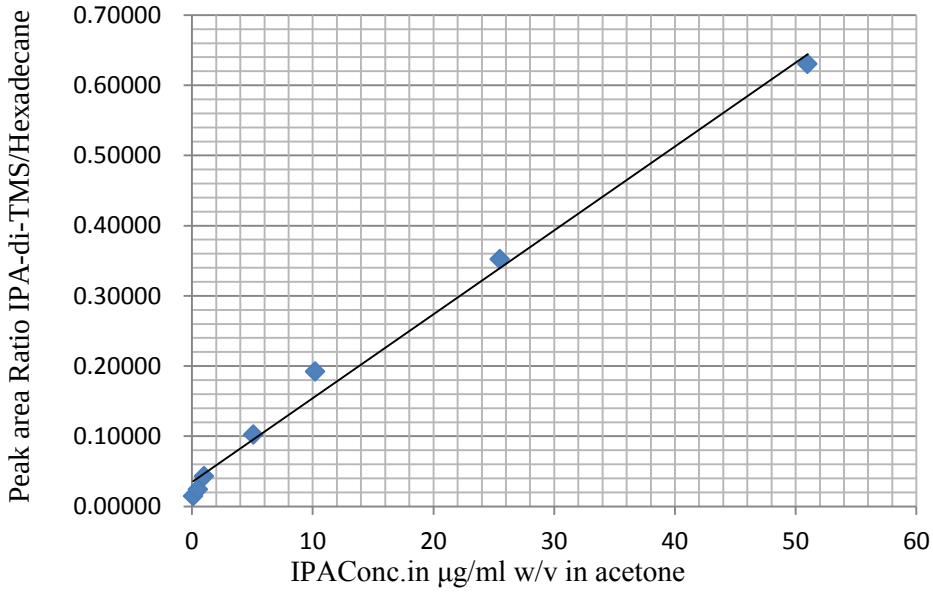


Figure 32: GC-FID NPG-di-TMS Calibration Curve

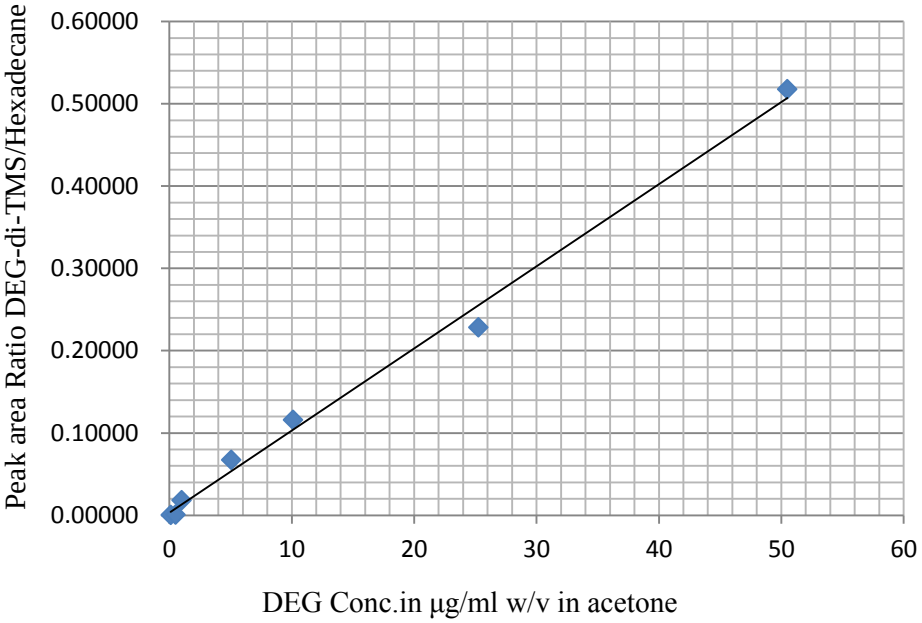


Figure 33: GC-FID DEG-di-TMS Calibration Curve

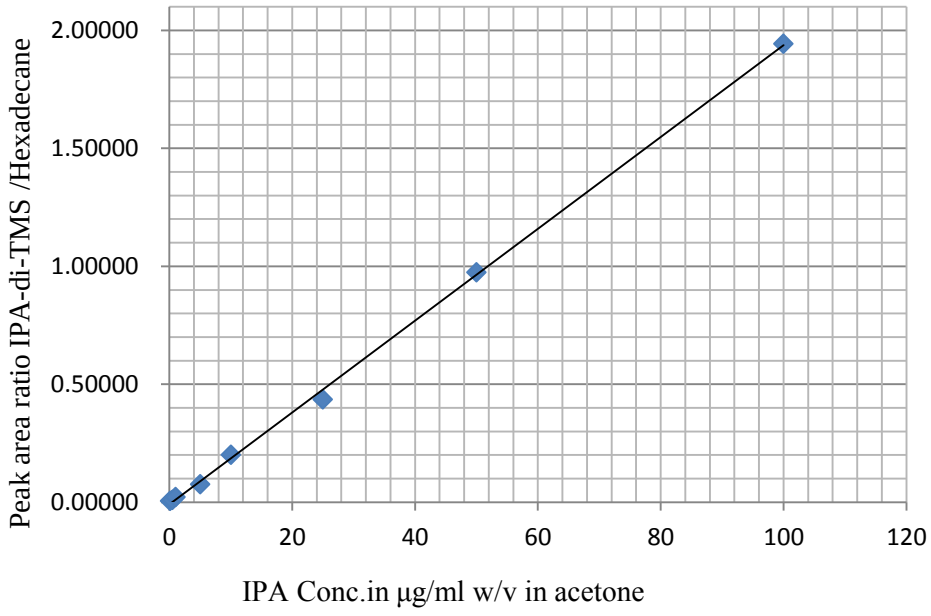


Figure 34: GC-FID IPA-di-TMS Calibration Curve

5.5 Cyclic Polyesters Hydrolysis by Human Liver S9 Fraction

The GC-MS chromatograms after hydrolysis of synthesized cyclic polyester standards by human liver S9 fraction are shown in Figures 35 to 37. Figure 35 and 36 indicate that DEG-AA and DEG-IPA were completely hydrolyzed after 60 min incubation with 2 mg/ml human liver S9 fraction. However, only DEG was found as a hydrolysis product, neither the diacid monomers nor the monoester intermediates were found. Figure 37 indicates that NPG-AA 2+2 was partially hydrolyzed to NPG and NPG-AA monoester; AA monomer was also not found in the hydrolysis products.

The disappearance of diacids may be due to their interactions with the complex human liver S9 fraction enzyme system. Previous research indicates that AA undergoes β-oxidation in rats and it decomposes to glutamic acid, lactic acid, β-ketoadipic acid and citric acid (Rusoff et al., 1960). IPA can also bind to amino acid residues close to the active site of some oxidase (Si et al., 2011). In our analysis we were not able to identify any metabolites that could arise from AA and IPA.

Based on these results, we suggest termination of the hydrolysis with shorter incubation time in order to investigate the intermediates of DEG-AA and DEG-IPA by human liver S9 fraction. An isotopic labeled AA, IPA and their corresponding cyclic oligoesters can be used for further investigation on the cyclic polyester metabolism pathway studies.

Our cooperate lab (Research Toxicology Centre S.p.A.) performed NPG-AA 2+2 *in vitro* Micronucleus Test in Chinese hamster V79 cells (Table 17). Their results showed that NPG-AA 2+2 induces micronuclei in Chinese hamster V79 cells after *in vitro*

treatment in the absence of rat liver S9 fraction, while no micronuclei are induced in the presence of S9 metabolism. By treating the cells with NPG-AA 2+2, marked increases in the number of micronucleated cells over the control value were observed at the high and intermediate dose levels (0.313mM and 0.256mM) in the absence of S9 fraction. Slight but not remarkable increases in the incidence of micronucleated cells over the control value were observed in the presence of S9 metabolism. Marked increases in the number of micronucleated cells were observed in cultures treated with the positive controls Cyclophosphamide, Mitomycin-C and Colchicine indicating the correct functioning of the assay system.

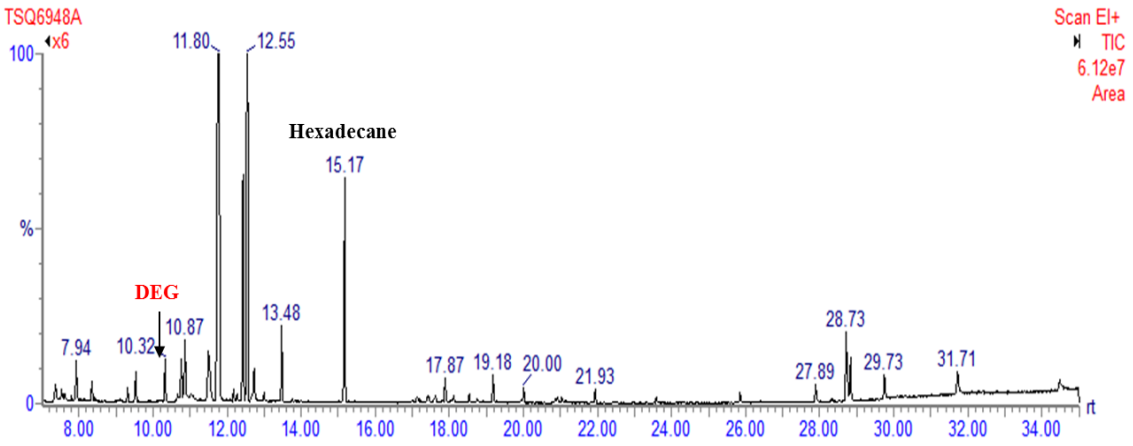


Figure 35: DEG-AA Hydrolysis with Human Liver S9 Fraction by GC-MS analysis

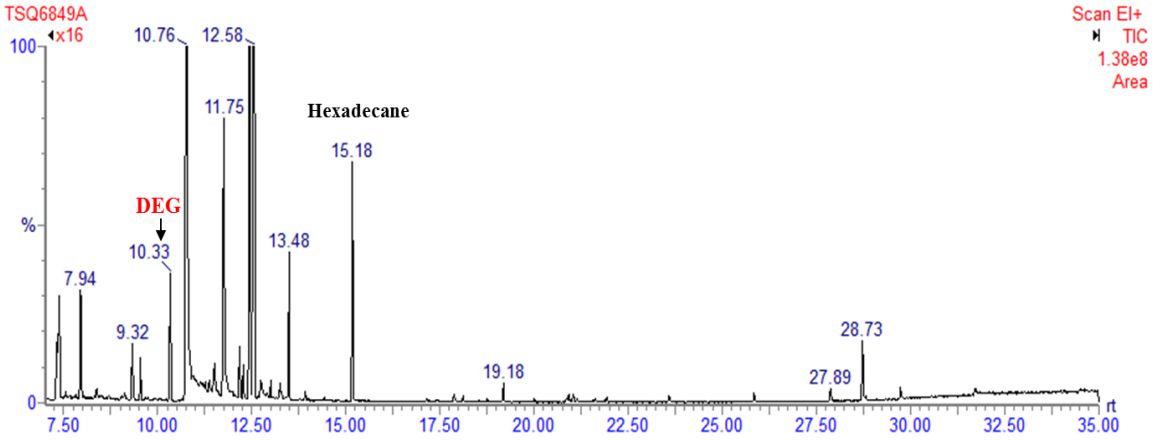


Figure 36: DEG-IPA Hydrolysis with Human Liver S9 Fraction by GC-MS analysis

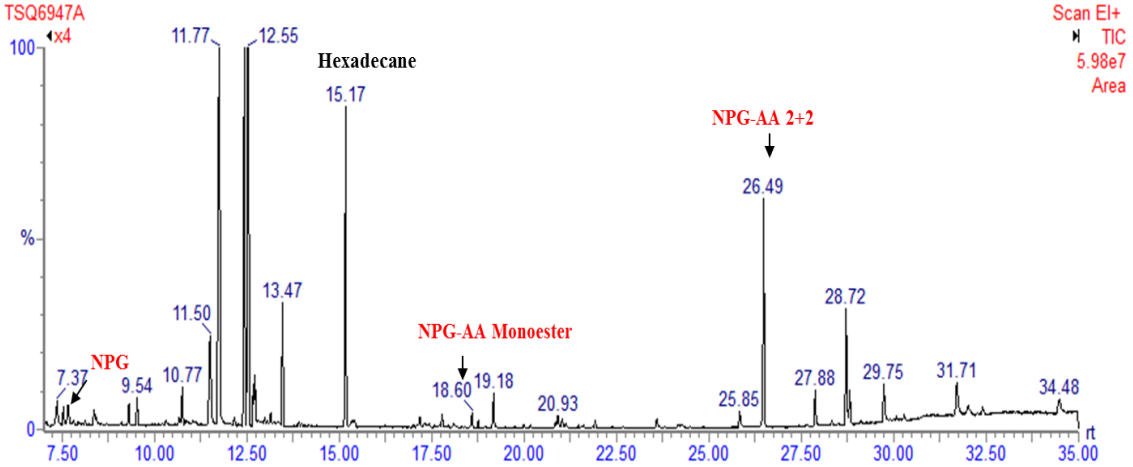


Figure 37: NPG-AA 2+2 Hydrolysis with Human Liver S9 Fraction by GC-MS analysis

Table 17: *In Vitro* Micronucleus Test in Chinese Hamster V79 Cells

Treatment	Dose Level (mM)	Presence of S9 Metabolism			Absence of S9 Metabolism		
		%Min Cells	Sig.	% Cytotoxicity	%Min Cells	Sig.	% Cytotoxicity
Untreated	0.0	0.85		4	0.80		3
Solvent 1%	0.0	0.75		0	0.45		0
NPG-AA 2+2	0.0781	-		-	0.85	NS	6
NPG-AA 2+2	0.156	-		-	7.05	***	37
NPG-AA 2+2	0.313	-		-	13.9	***	51
NPG-AA 2+2	0.625	1.10	NS	-1	-		-
NPG-AA 2+2	1.25	1.00	NS	4	-		-
NPG-AA 2+2	2.50	1.15	NS	7	-		-
Mitomycin-C	0.30 µg/ml	-		-	5.75	***	10
Colchicine	2.00 µg/ml	-		-	2.20	***	-22
Cyclophosph -amide	10.0 µg/ml	4.50	***	59	-	-	-

Note: %Mn Cells: Percentage of cells bearing micronuclei
Sig.: Statistical significance
NS: Not significant
- : Not tested or not selected for scoring
***: Statistically significant at $p<0.001$

5.6 *In Vivo* Metabolites of NPG-AA 2+2 from Mouse Plasma

Figure 38 shows the PLE hydrolysis products detected by GC-MS in SIM mode for maximum sensitivity. Figure 39 shows examples of the mouse plasma products detected by SIM mode.

The GC-MS analysis data of the mouse plasma samples is shown in Table 17. The data indicate that little or no cyclic-NPG-AA 2+2 was present in the metabolites. However, the two metabolites NPG and AA were clearly detected and confirmed by full scan mass spectra and their concentration appeared to be dose dependent. NPG-AA 1+1 open ring structure was not detected in any of the blood samples, presumably because of its decomposition.

The concentrations of metabolites appeared to be dependent on dose and metabolism time (Table 18). For group 6 with 0 mg/kg dose, NPG, AA and cyclic NPG-AA 2+2 were not present. For group 7A and 7B with 500 mg/kg dose, the mean NPG metabolites concentrations were 2.63 μg/ml and 2.78 μg/ml; the average AA metabolite concentrations were 0.85 μg/ml and 0.71 μg/ml. For 8A and 8B with 1000 mg/kg dose, the mean NPG metabolite concentrations were 12.75 μg/ml and 10.92 μg/ml; the mean AA metabolite concentrations were 4.51 μg/ml and 0.65 μg/ml. For group 9A and 9B with 1500 mg/kg dose, the mean NPG metabolite concentrations were 16.89 μg/ml and 9.76 μg/ml; the mean AA metabolite concentrations were 3.83 μg/ml and 0.71 μg/ml. In general, for Group B (after 30 min metabolism) the average concentrations of NPG and AA recovered were less than those of Group A (after 15 min metabolism) (Figure 40).

These results are consistent with previous *in vitro* PLE and human liver S9 fraction studies showing the NPG-AA 2+2 susceptibility to enzyme hydrolysis. It also accounts for the lack of genotoxicity of NPG-AA 2+2 in the *in vitro* micronucleus test. The results verify that cyclic-NPG-AA 2+2 can be rapidly and completely hydrolyzed in mice.

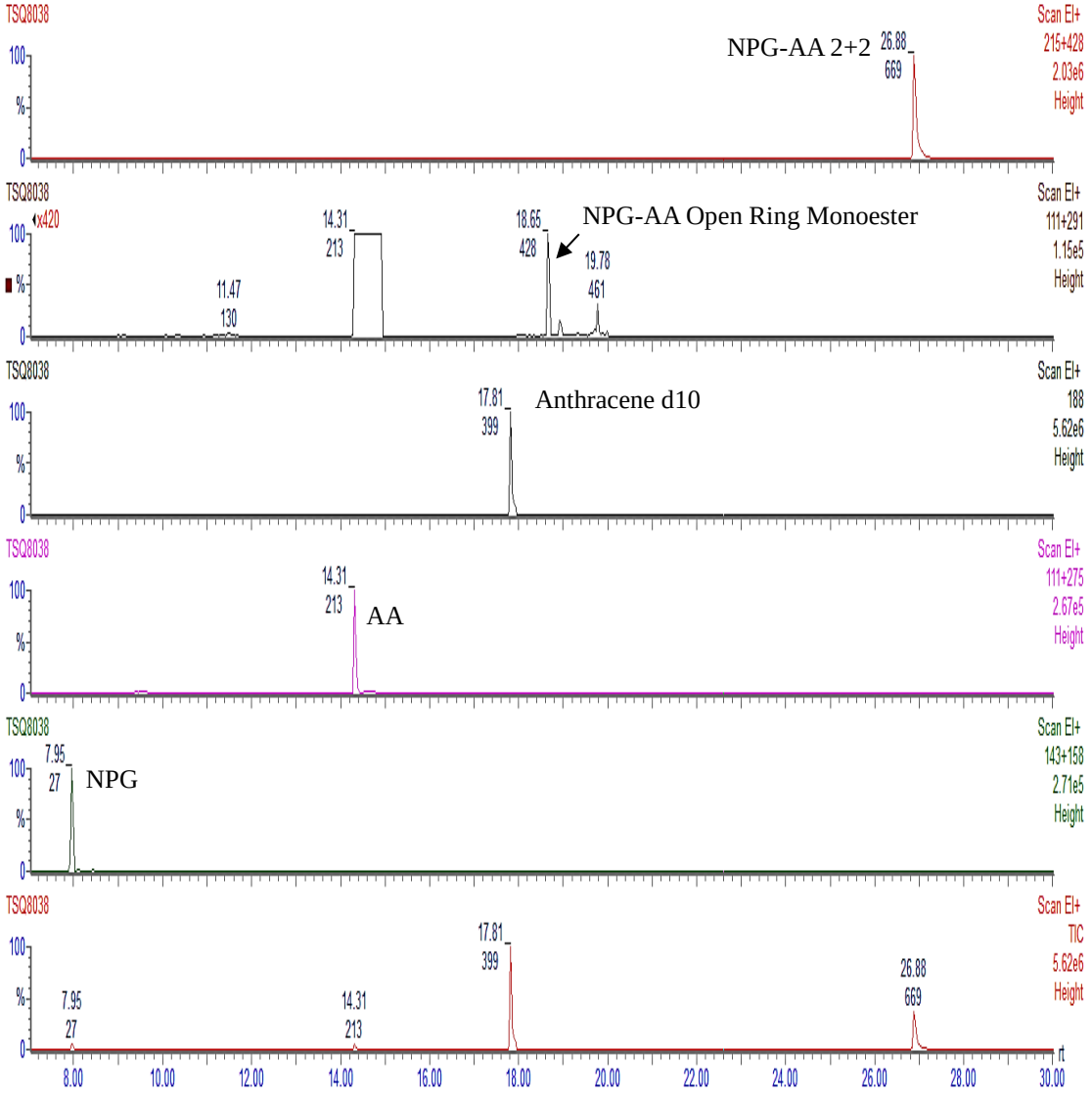


Figure 38: NPG-AA 2+2 PLE Hydrolysis Products Detected by GC-MS SIM (Anthracene-d_{10} Internal Standard Detected)

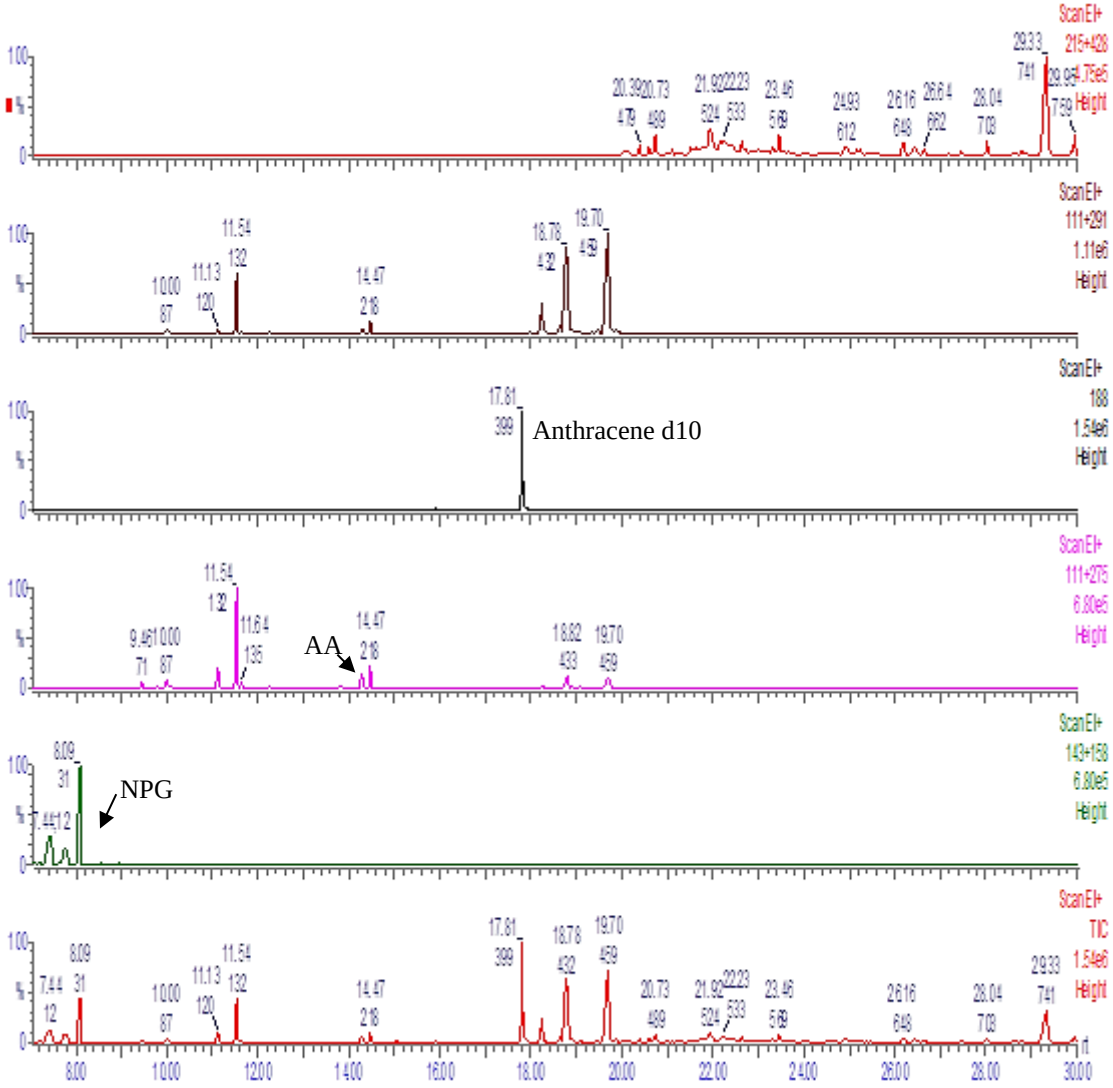


Figure 39: Mouse 41 Group 7A Plasma TMS Derivatives Detected by GC-MS SIM (Anthracene-d_{10} Internal Standard)

Table 18: GC-MS Analysis Results for Cyclic-NPG-AA 2+2 Metabolites in Mouse Plasma

Animal Number	Group	¶NPG (µg/ml)	¶AA (µg/ml)	NPG-AA open ring structure (µg/ml)	‹Cyclic NPG-AA-2+2 (µg/ml)
36	6	none detected	none detected	none detected	none detected
37	6	none detected	none detected	none detected	none detected
38	6	none detected	none detected	none detected	none detected
39	7A	2.84	1.11	none detected	0.03
40	7A	1.2	0.36	none detected	0.03
41	7A	3.86	1.08	none detected	0.04
42	8A	13.47	8.12	none detected	0.1
43	8A	15.24	3.06	none detected	0.03
44	8A	9.53	2.34	none detected	0.03
45	9A	17.09	1.59	none detected	0.11
46	9A	10.4	1.98	none detected	0.05
47	9A	23.19	7.93	none detected	0.04
48	7B	1.66	0.16	none detected	0.02
49	7B	3.14	0.5	none detected	0.05
50	7B	3.53	0.52	none detected	0.06
51	8B	6.32	0.67	none detected	0.04
52	8B	11.26	0.7	none detected	0.03
53	8B	15.18	0.58	none detected	0.02
54	9B	12.06	0.82	none detected	0.11
55	9B	12.64	0.17	none detected	0.19
56	9B	4.6	0.15	none detected	0.04

Note: ¶NPG and AA were both confirmed present in the plasma by full-scan MS. The peaks for these 2 metabolites were well chromatographically resolved and had good signal to noise ratio (s/n).

‹Cyclic-NPG-AA-2+2 was at or near detection threshold, the peak had poor s/n ratio and was not reliably confirmed by full scan MS

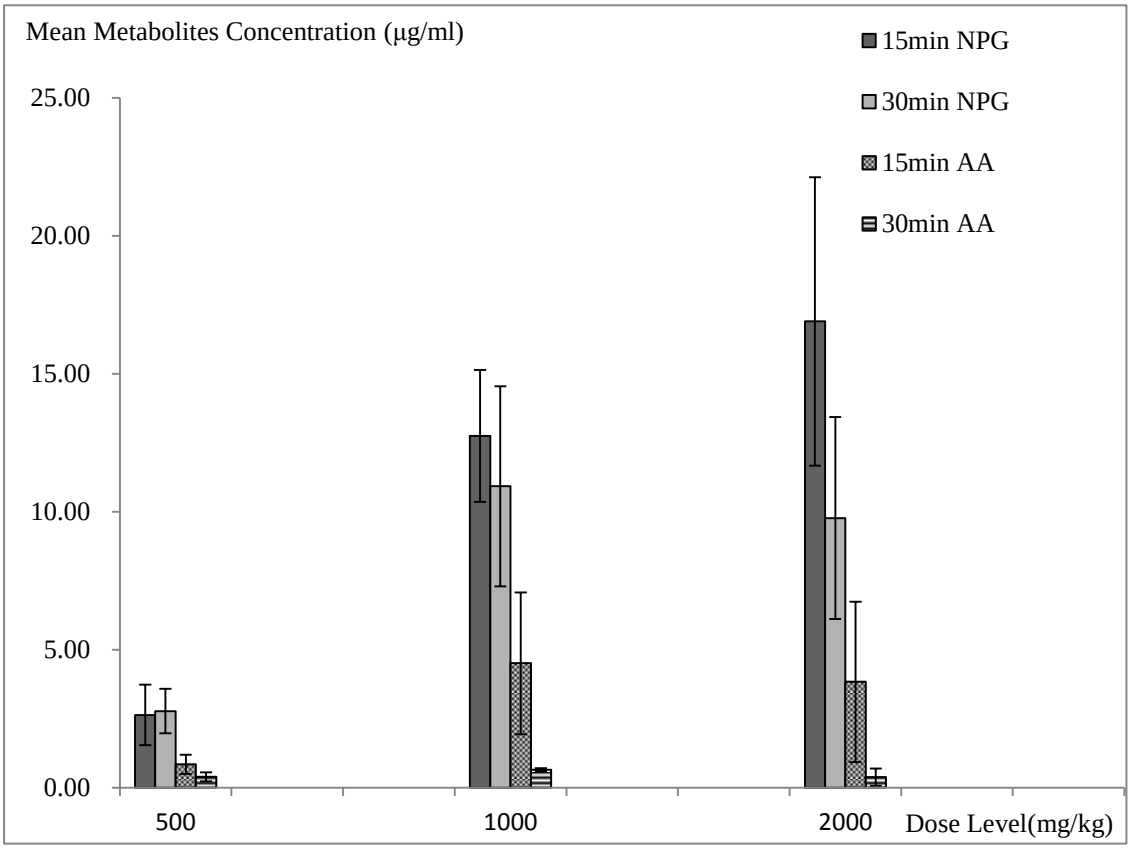


Figure 40: Mouse Plasma Cyclic-NPG-AA 2+2 Metabolites Concentrations from Different Dose Levels and Harvest Time Points

6 CONCLUSION

From our accumulated reports on food packaging laminates migration studies over the years, 23 new short chain cyclic polyesters and 4 linear chain ester migrants were identified. The major mass spectral fragments of these compounds from GC-MS (EI) analysis were summarized and their fragmentation were presented and discussed.

The prevalence of the currently identified short chain ester migrants was summarized based on their frequency of occurrence in laminated packaging. The results indicate that DEG, NPG, AA and IPA based ester migrants has a relative higher occurrence rate than the others, thus we chose the four most prevalent cyclic oligoesters-DEG-AA, DEG-IPA, NPG-AA and NPG-AA 2+2 as representative migrant types for our metabolism studies.

The PLE hydrolysis study on synthesized authentic standards of DEG-AA, DEG-IPA and NPG-AA 2+2 indicates that these cyclic esters can be hydrolyzed in a stepwise process with the formation of their respective open ring intermediates followed by a complete scission to their monomers. Different types of cyclic polyesters also showed different resistance to hydrolysis. Aliphatic type cyclic ester migrant DEG-AA was less resistant than the aromatic type cyclic ester migrant DEG-IPA, while dimer type NPG-AA 2+2 was even more resistant than DEG-IPA.

The PLE hydrolysis study on actual laminate extracts with NPG-AA and NPG-AA 2+2 or MP-diol-AA and MP-diol-AA 2+2 indicates that these cyclic esters can be completely metabolized into the monomers.

Enzyme concentration, surfactant addition and incubation time all played important roles in the cyclic oligoester migrants' hydrolysis. The rate of hydrolysis increased with enzyme concentration, incubation time and the presence of a surfactant.

Calibration curves of TMS derivatized AA, IPA, NPG and DEG were constructed for the quantification of the cyclic oligoester monomer metabolites. This will indirectly help with the toxicity evaluation of the migrants because the toxicity information of the monomer metabolites is much more abundant.

The human liver S9 fraction hydrolysis study of synthesized authentic standards of DEG-AA, DEG-IPA and NPG-AA 2+2 showed that these cyclic esters can be metabolized in human liver. As with the PLE hydrolysis results, DEG-AA and DEG-IPA were metabolized more readily than NPG-AA 2+2. The acid monomer metabolites were not detected in the hydrolysis products presumably because of their possible reactions with the other enzymes present in the human liver S9 fraction.

Finally, the GC-MS analysis results of the *in vivo* metabolism study of NPG-AA 2+2 shows that NPG-AA 2+2 can be metabolized (or hydrolyzed) to AA and NPG in mice. The levels of metabolites detected varied with the dose and digestion time.

Overall, we provided analytical methods and identification information on the cyclic oligoester migrants that are of great concern to the food packaging industry and regulatory authority. By investigating the metabolism of the most prevalent and representative cyclic oligoester migrants, we were able to assist in establishing the risk assessment of the cyclic oligoesters which could form a basis for future legislation and scientific research.

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