

# Assessing Leaching of Clear Aligners in Saliva via Mass Spectrometry

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*2023 Research Aid Awards (RAA)*

*Dr. Ronnel Azizollahi*

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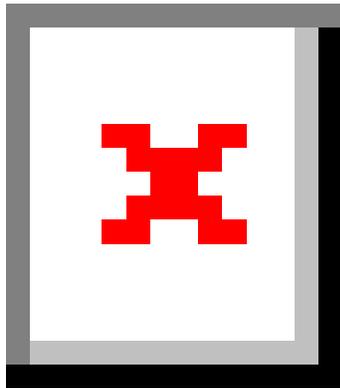
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## Institution

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## Names of principal advisor(s) / mentor(s), co-investigator(s) and consultant(s)

Christine Hong, DMD, MS; Kara Lynch, PhD

## Amount of Funding

\$6,000.00

## Abstract

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Clear aligners have become a commonly used and sought-after appliance within orthodontics to correct malocclusion. Current literature is inconsistent in the biocompatibility of thermoplastic and 3D printed clear aligner systems, with a lack of in-vivo studies. This study aims to investigate whether clear aligners leach substances, attempting to replicate in-vivo conditions, in efforts to ensure the safety of and protect the quality of the appliances patients are offered.

The objective of this study is to assess release of plastic components, including monomers of plastic polymers and plasticizers used to make plastic flexible, in salivary samples from clear aligner trays in-vitro as well as to assess release of substances in salivary samples obtained from patients after wearing clear aligners at different time points as an in-vivo analysis. The hypothesis is that clear aligners leach cytotoxic plastic components into saliva with high concentrations upon first insertion and less leaching occurs over time.

In our preliminary studies, non-used clear aligners were incubated in saliva and tested using mass spectrometry. We identified caprolactam, suggesting that invisalign aligners are predominately made of a polycaprolactam polymer. Caprolactam is a compound that has been reported to have adverse effects upon contact. From existing literature assessing leaching of substances from resins in restorative material, compounds become less prevalent in saliva over time, so we expect similar results, likely due to dilution effects as we stimulate more salivary creation over time.

Although clear aligners have become a popular tool in orthodontics due to esthetics and improved oral hygiene maintenance, and less frequent appointments, there have been adverse effects reported by users and therefore it is important to evaluate the safety of this appliance 1.

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AAOF funding has played a crucial role in furthering my career by supporting my research on the biocompatibility of clear aligners—a novel and increasingly relevant area in orthodontics. This project not only contributed to the advancement of scientific understanding in the field, but also enabled me to pursue a Master of Science degree in Oral and Craniofacial Sciences alongside my clinical training. The funding has also enriched my academic experience by providing opportunities for teaching and mentorship. I had the chance to guide a predoctoral student through aspects of the research process, which strengthened my leadership skills and deepened my commitment to academic dentistry. Looking ahead, this experience has laid a strong foundation for my long-term goal of pursuing a career in academia, where I hope to continue contributing to research, teaching, and mentoring the next generation of orthodontic professionals.

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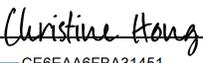
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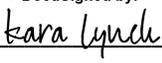
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in the  
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of the  
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# Assessing the Leaching of Clear Aligners in Saliva via Mass Spectrometry

Ronnel Azizollahi

## ABSTRACT

Clear aligners are increasingly popular in orthodontics due to their aesthetic appeal, patient comfort, and multifunctional use as retainers, whitening trays, and splints. However, their prolonged intraoral exposure raises questions about the biocompatibility of the polymers used in their manufacture. This study investigates the potential release of caprolactam—a known monomer used in plastic production—from Invisalign® aligners into human saliva. Using both in vitro and in vivo methods, salivary samples were analyzed via high-resolution mass spectrometry to assess compound leaching at multiple time points. In-vitro data demonstrated a time-dependent increase in caprolactam concentration over a two-week incubation period, while in-vivo samples revealed a peak at one hour post-insertion, followed by a decline toward baseline. A population-based analysis explored correlations between salivary caprolactam levels and variables including age, treatment duration, and daily wear time, revealing no significant associations. Although the detected concentrations were below established toxicological thresholds, the consistent presence of leached compounds highlights a need for ongoing scrutiny. This study contributes to the growing body of literature on the safety of clear aligner systems and emphasizes the need for transparent material disclosure and standardized biocompatibility testing in orthodontic care.

# Table of Contents

1. INTRODUCTION.....	1
1.1 Preface .....	1
1.2 Preliminary Study.....	4
2. HYPOTHESIS .....	7
3. SPECIFIC AIMS.....	8
4. MATERIALS AND METHODS.....	9
4.1 Funding.....	9
4.2 In-vitro Experimental Design.....	9
4.3 In-vivo Experimental Design.....	10
4.4 Population Study Experimental Design.....	12
4.5 Sample Preparation.....	13
4.6 Chromatography and Mass Spectrometry Analysis .....	13
5. RESULTS.....	15
5.1 In-vitro Results.....	15
5.2 In-vivo Results .....	16
5.3 Population Study Results.....	16
5.3.1. Quantifying Caprolactam in a population of users .....	17
5.3.2 Qualitative Analysis of Contaminants .....	20
6. DISCUSSION.....	27

7. CONCLUSION.....31

REFERENCES .....32

## List of Figures

Figure 1. Chromatographic peaks for compounds identified in Invisalign Trays via GC-HRMS ..5	
Figure 2. In-vitro Experimental Design .....	10
Figure 3. In-vivo Experimental Design.....	12
Figure 4. Population Study Experimental Design .....	13
Figure 5. Caprolactam concentration at incubation time points 1h, 4h, 2h, 1wk, 2wks .....	15
Figure 6. Caprolactam concentration of four patients followed over 2 weeks for four timepoints .....	16
Figure 7. Correlation graphs between concentration of caprolactam and age, TSS, and DWT ....	19
Figure 8. Compounds identified in more than 60% of the 31 patients in the population study ....	21

## List of Tables

Table 1. Assessing associations between age, time since started treatment (months), daily wear time (hours), and concentration of caprolactam.....	16
Table 2. Significance of spearman’s rho for concentration of caprolactam and age, TSS, and DWT.....	19
Table 3. Industrial Applications and Toxicological Profile of Compounds in > 60% of Samples .....	22

## List of Abbreviations

DWT: Daily Wear Time

GC-HRMS: Gas Chromatography High Resolution Mass Spectrometry

HGFs: Human Primary Gingival Fibroblasts

LC-HRMS: Liquid Chromatography High Resolution Mass Spectrometry

MNPs: microplastics and nanoplastics

TSS: Time Since Start of Treatment

## 1. INTRODUCTION

### 1.1 Preface

Clear aligners, which are a series of plastic removable trays, have become a commonly used and sought-after appliance within orthodontics to correct malocclusion. Aligners have become a booming appliance in orthodontics in large part because of a “significant increase in the esthetic demands of the patients” [1]. Beyond orthodontic tooth movement, clear aligners are used as retainers, whitening trays, and splints for temporomandibular joint disorders [2].

Several advantages make clear aligners appealing to both patients and providers. For patients, the removability of aligners allows for easier maintenance of oral hygiene and greater comfort during meals [3]. From the provider’s perspective, clear aligner therapy reduces chair time, enables digital treatment monitoring, minimizes the frequency of appointments and emergencies, and requires less clinical assistance during procedures such as bonding[4].

Despite these benefits, adverse events associated with clear aligner use have been reported. In a retrospective analysis of the FDA’s Manufacturer and User Facility Device Experience database, Allareddy et al. identified multiple user-submitted reports detailing symptoms such as “difficulty breathing, sore throat, swollen throat, swollen tongue, hives and itchiness, anaphylaxis, swollen lips, and feeling of throat closing tight” among several other adverse symptoms[5].

Amid growing public concern regarding the health effects of microplastics and nanoplastics (MNPs), researchers have turned attention to orthodontic appliances. A recent study found that patients with MNPs detected in their carotid atheromatous plaques had an elevated risk of myocardial infarction, stroke, or death [6]. This underscores the need to examine the long-term health impacts of clear aligners and the substances they may release.

Aligners are typically recommended by orthodontists to be worn for up to 22 hours per day and changed every 1–2 weeks to achieve tooth movement, meaning they remain in contact with the oral environment for extended periods. This raises questions about possible interactions between the aligner material and the oral cavity that could affect both tray durability and human health. Notably, internet searches noted a large “increase in searches for ‘Invisalign’ at the beginning of 2021” [7]. With the increase in patients using clear aligners, we have also seen an increase in patients that have developed allergies or reactions to the aligners, especially since the coronavirus pandemic in 2019.

As discussed with the increased search queries, the most popular brand in the market has been Invisalign which uses its trademarked *SmartTrack* material that is made of a thermoplastic polymer [8]. The thermoplastic polymers include “polyurethane and co-polyester” [9]. The Invisalign website virtual chat function states that their aligners “contain no BPA, BPS, latex or gluten”; however, the full composition of Invisalign’s proprietary aligners are not disclosed. Many other thermoplastic aligners, that have disclosed their components, are made of polyesters, polyurethane, polypropylene, polyethylene terephthalate glycol, polycarbonate, ethylene vinyl acetate, and more [10]. Polyurethane can be affected by features of the oral cavity such as heat, moisture and salivary enzymes[9]. This interaction can cause the release of molecules that can be harmful to humans on a cellular level [11]. The thermoformed process used to create aligners is linked to cytotoxicity, but the curing process tends to reduce this toxicity[12]. It has also been noted that increased post-processing can reduce the mechanical strength of aligners [13].

Aligners vary in material composition, manufacturing processes, and additives. Alhendi et al. tested four brands—Invisalign®, Eon®, SureSmile®, and Clarity®—by immersing them in ethanol solutions of varying concentrations. Substances such as benzene 1,3-bis(1,1-

dimethylethyl), phenol derivatives, and other potentially harmful compounds were identified. Invisalign released the least number of chemicals, primarily benzene derivatives in high ethanol concentrations [2] [14]. However, ethanol solutions do not replicate the human oral environment. Our study aims to address this by incubating aligners in saliva, a clinically relevant and reliable, non-invasive biomarker.

In another study by Martina et al. the cytotoxicity of four different clear aligner materials (Duran, Biolon, Zendura, and SmartTrack) were assessed on human primary gingival fibroblasts (HGFs) [11]. HGFs are the primary cells present in intra-oral tissues and are therefore the cells most exposed to aligners in the body [15]. Both the thermoformed and non-thermoformed materials were tested, except the SmartTrack material was only tested as thermoformed. The samples were incubated in a medium at 37°C for 14 days, after which they all showed slight cytotoxicity to HGFs, with the thermoformed materials showing higher cytotoxicity, and the highest was in Biolon, Zendura, SmartTrack, and then Zendura with the lowest cytotoxicity [11]. In a similar study, 3D printed aligners were left in sterile deionized water for 14 days and the cytotoxicity and estrogenicity of released factors were investigated with assays on human gingival fibroblasts and breast cancer cell lines, but there was no significant cytotoxicity nor estrogenicity found [16].

In a study recently published in February of 2025 investigating both the toxicity of both Hawley and Essix retainers, Turkish researchers found an increase in indicators of oxidative damage to DNA in the Hawley retainers group; conversely they also found higher levels of cell damage in the Essix retainers group after 2 to 3 weeks of wear, although not reporting clinical significance [17]. This conveys that further investigation into substances released by orthodontic

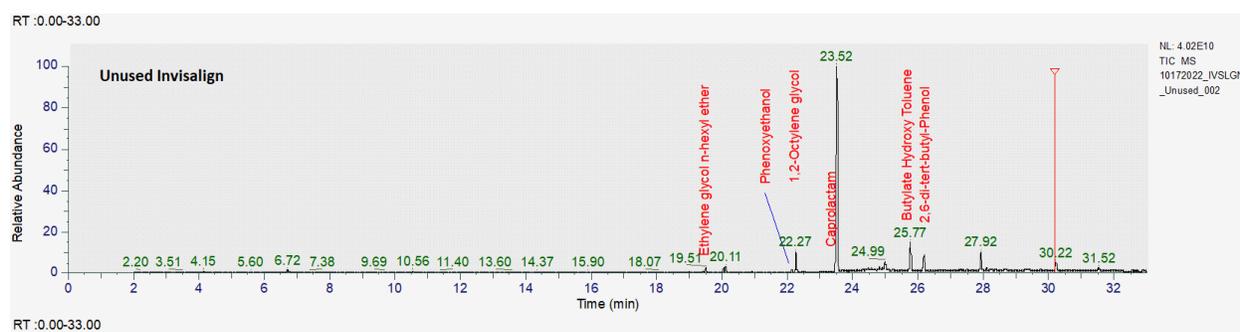
appliances used by patients should be continued to develop a deeper understanding of long term health effects.

Retainer cleaning methods may also influence material integrity. Wible et al. used scanning electron microscopy to assess copolyester and polypropylene/ethylene copolymer retainers cleaned with various solutions. They aimed to assess differences in light transmittance, surface roughness and flexural modulus of various copolyester and polypropylene/ethylene copolymer retainers subjected to different cleaning methods including: Invisalign cleaning crystals, RetainerBrite, Polident, Listerine mouthwash, 2.5% acetic acid, 0.6% NaClO, and 3% H2O2. Over six months, no significant degradation was observed, suggesting these materials maintain physical stability with typical cleaning methods [18] [19].

## 1.2 Preliminary Study

To identify target compounds for our analysis, a preliminary study was conducted in the Lynch Laboratory at San Francisco General Hospital. An unused Invisalign tray and a non-thermoformed in-house tray (Great Lakes Dental Technologies Thermal Forming Clear Splint Biocryl 1mm) were evaluated directly using a Gas Chromatography (GC) High Resolution Mass Spectrometer (HRMS) to assess the composition. Briefly, a GC-HRMS system that was equipped with a thermal desorption unit was used to analyze a small piece of each tray directly. The plastic trays were heated to 100°C and cryo-focused at -150°C in the thermal desorption unit and then analyzed by HRMS in order to determine candidate residual monomers and plasticizers used in the manufacture of the trays. The analysis revealed higher levels of caprolactam, a synthetic monomer used in plastic production, in Invisalign compared to the non-thermoformed in-house tray. Other Invisalign tray components identified include, ethylene glycol-n-hexyl ether, phenoxyethanol, 1-2-octylene glycol, butylated-hydroxytoluene, and 2,6-di-tert-butyl-phenol.

These components range in function from plasticizers, solvents in resins used for polymer generation, germicides, to antioxidant for plastics that prevent thermal degradation. Figure 1, shows the chromatographic peaks for the Invisalign constituents as detected using the GC-HRMS system. These preliminary results are promising, however, alternative mass spectrometric approaches were needed to provide a comprehensive analysis of all constituents. We aimed to further assess whether these compounds or their breakdown products leach into saliva, with caprolactam being our compound of interest.



**Figure 1.** Chromatographic peaks for compounds identified in Invisalign Trays via GC-HRMS

Caprolactam is absorbed through skin or inhalation, metabolized in the liver, and excreted primarily in urine [20] [21]. While the exact metabolic pathways aren't fully documented in humans, research in animals shows that caprolactam is quickly cleared, usually within a day or two, indicating a short biological half-life [21]. Short-term exposure to high levels of caprolactam can cause irritation in the eyes and skin, and cause symptoms such as headaches, nausea, and dizziness [22]. It can also irritate respiratory mucous membranes at 5 ng/mL while also having adverse effects on the central nervous system lymphocytes of the liver, and the menstrual cycle and reproductive system of female workers[23] [24] [25] [26]. Long-term exposure in industrial settings may lead to more serious health effects [27]. Some studies on

caprolactam, such as one by Unger et al. in 1981, have explored its metabolism and excretion in rodents, finding that ~80% of radiolabeled caprolactam was excreted in urine after 24 hours with a half-life of 3 hours in the bloodstream. It also found that caprolactam was well distributed in body tissues but had low retention in adipose tissue [28].

Though non-bioaccumulative, it has been shown to act as an androgen receptor antagonist in environmental studies [29]. These results warrant further research into its effects on the human body and its possible endocrine disrupting effects [29]. Other case reports in China noted symptoms such as seizures after heavy exposure to caprolactam, as well as insomnia, nosebleeds, and infertility [20]. Caprolactam also serves as a monomer in polyamide 6, a material widely used in food packaging. There is also a potential for the migration of polyamides into food upon contact with factors such as packaging composition, contact time, food type, irradiation and temperature playing a role [30].

Current literature is inconsistent in the biocompatibility of clear aligner systems, with a lack of in-vivo studies. There have also been conflicting results published, as well as an incessant release of new aligner brands on the market that should be investigated for safety [31]. The systemic effects of clear aligners are unknown because of the few studies conducted thus far [12]. This study aims to investigate whether clear aligners leach toxins, attempting to replicate in-vivo conditions, in efforts to ensure the safety of and protect the quality of the appliances patients are offered for orthodontic treatment.

## 2. HYPOTHESIS

The hypothesis is that clear aligners leach potentially harmful plastic components into saliva with high concentrations upon first insertion and less leaching over time. From existing literature that investigates leaching of substances from resins in restorative material, compounds become less prevalent in saliva over time, so we expect similar results, likely due to dilution effects as we stimulate more salivary creation over time [7].

### 3. SPECIFIC AIMS

AIM #1 In-vitro: Assess release of caprolactam (as a representative of potential harmful plasticizers) in salivary samples from clear aligner trays in-vitro.

AIM #2 In-vivo: Assess release of caprolactam in salivary samples obtained from patients after wearing clear aligners at different time points: in-vivo analysis.

AIM #3 Population study: Assess whether there is an association between age, time since start of treatment in months, hours of wear per day, and concentration of caprolactam in salivary samples.

## 4. MATERIALS AND METHODS

### 4.1 Funding

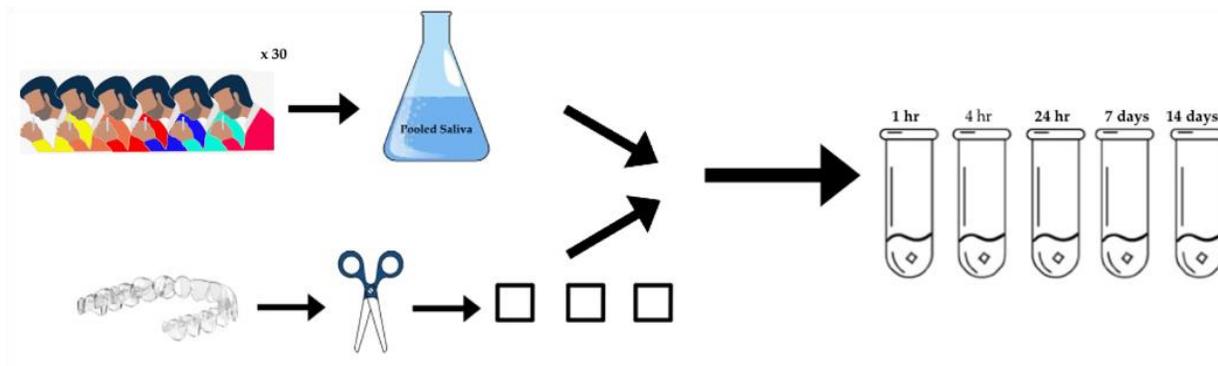
The American Association of Orthodontists Foundation provided funding for this project through the Research Aid Award.

### 4.2 In-vitro Experimental Design

Unstimulated whole salivary samples were collected from each patient that meets inclusion criteria. The inclusion criteria include ages 18-40. The exclusion criteria include past orthodontic treatment (including the use of retainers and nightguards), restorative dental work within the past year, use of chlorhexidine mouth rinse, clinically evident clenching/grinding habits, pregnancy or breastfeeding, and salivary gland dysfunction. Patients were debriefed on requirements of the study and asked to sign a consent form to agree to participation. Patients were asked not to eat or drink for at least 1.5 hours before saliva collection [32].

3mL of saliva was collected in a glass tube per patient. Salivary samples were pooled from all pre-treatment subjects (sample size n=24) and mixed thoroughly by agitation and sonication to break surface tension and prevent foam formation. Pooled saliva was frozen at -80 C to maintain salivary components and was thawed to average body temperature of 37 C before performing next steps. 1 mL of pooled saliva was placed in borosilicate glass lined eppendorf tubes using glass pipettes and were agitated again with the vortex genie mixer. Invisalign® aligners were cut with scissors into 5x5mm pieces. There were 5 eppendorf tubes that correlated to the amount of time the aligner was soaked in the pooled saliva before mass spectrometry analysis: 1 hour, 4 hours, 24 hours, 7 days, and 14 days. These time points allowed us to reproduce the treatment timeline by testing the entire range from first insertion to changing aligners at one to two weeks in. There were three samples run for each of the above five

timepoints. Aligners were placed in each tube and incubated for the respective time. Aligner pieces were removed after the indicated incubation time with SS tweezers which were cleaned between each removal. Acetonitrile was added to the respective tube which was then vortex mixed and centrifuged at a speed of 3000 for 10 minutes. The acetonitrile layer was removed and dried down with nitrogen evaporation not exceeding 80 psi for 20 minutes. Nitrogen is inert and does not oxidize the sample. 100  $\mu$ L of specimen prep buffer which is water, 10% methanol-ACN with 0.01% formic acid in a 1 to 9 ratio was placed in the respective tube which was again vortex mixed. The solution was then transferred into amber vials with glass inserts and the LC-HRMS was ran.



**Figure 2.** In-vitro Experimental Design

#### 4.3 In-vivo Experimental Design

The inclusion criteria for the in-vivo study include ages 18-40. The exclusion criteria include past orthodontic treatment (including the use of retainers and nightguards), restorative dental work within the past year, use of chlorhexidine mouth rinse, clinically evident clenching/grinding habits, pregnancy or breastfeeding, and salivary gland dysfunction. Patients were debriefed on requirements of the study and asked to sign a consent form to agree to

participation. Participants were asked if they had been wearing the aligners as instructed at each visit. If the patient agreed, an unstimulated whole saliva sample was collected. If a participant was not compliant with instructed use, they were disqualified from the study. Pre-treatment saliva was collected from a patient who fit the inclusion criteria and had not started treatment yet. This was time point T0. We only investigated Invisalign® clear aligners. Clear aligners and respective attachments were delivered to the patient and saliva was collected from the patient one hour after placement; this was T1. Saliva was then collected from the patient 1 week after aligner delivery which was T2 and 2 weeks after aligner delivery which was T3. T3 salivary sample was taken before the patient was given the next set of clear aligners in the treatment sequence. 3mL of saliva was collected in a glass tube per patient at each time point. All salivary samples for the respective patient were collected around the same time (+/- 1 hour). For example, if patient X's T0 was collected at 9am, then T1, T2, and T3 were also collected at 9am (+/- 1 hour). The sample size for the in-vivo analysis was n=4. Participants were given \$125 gift cards at T3 if they completed salivary collection at T0, T1, T2, T3 for incentive to complete participation. Acetonitrile was added to the respective tube which was then vortex mixed and centrifuged at a speed of 3000 for 10 minutes. The acetonitrile layer was removed and dried down with nitrogen evaporation not exceeding 80 psi for 20 minutes. Nitrogen is inert and does not oxidize the sample. 100 µL of specimen prep buffer which is water, 10% methanol -ACN with 0.01% formic acid in a 1 to 9 ratio was placed in the respective tube which was again vortex mixed. The solution was then transferred into amber vials with glass inserts and the LC-HRMS was run. We identified compounds that were detected at time-points T1, T2, and T3 and not at T0. We compared the relative abundance of compounds detected at each time point. The identified

compound that was leached in highest levels in saliva (caprolactam) was then quantified by using an analytical standard of those caprolactam to generate calibration curves.



**Figure 3.** In-vivo Experimental Design

#### 4.4 Population Study Experimental Design

We assessed the release of caprolactam in salivary samples obtained from patients currently in aligner treatment, noting age, time since start of treatment (TSS) in months, and daily wear time (DWT) in hours. The inclusion criteria for the in-vivo study include ages 18-40.

Saliva samples were collected from n=31 patients spanning a range of ages and durations of aligner use in terms of DWT and TSS as noted in Table 1. To ensure robust comparisons, we included blanks from a pool of patients without orthodontic treatments, as well as synthetic saliva controls to serve as controls. Liquid phase extraction was followed by analysis with Gas Chromatography High Resolution Mass Spectrometry (GCHRMS) using both the Agilent E&L library and NIST library.



**Figure 4.** Population Study Experimental Design

#### 4.5 Sample Preparation

All extractions were conducted in glassware to minimize any plastics from pipette tips and centrifuge tubes. Two blanks were used: synthetic saliva and saliva collected from the in-vitro study. Blanks were processed exactly like samples as follow:

1 mL saliva was measured in a glass tube and extracted with 3 mL chilled HPLC grade acetonitrile spiked with internal standard (Fentanyl-d5 at 100 ng/mL). The tube was then centrifuged at 3000 rpm for 15 minutes. Then 1 mL of the supernatant was dried down under nitrogen stream. Samples analyzed by GC-qTOF were resuspended in 100  $\mu$ L solvent of methanol and ethyl acetate (9:1). Samples analyzed by LC-HRMS were resuspended in 100  $\mu$ L solvent of water and methanol (9:1) at 0.1% formic acid.

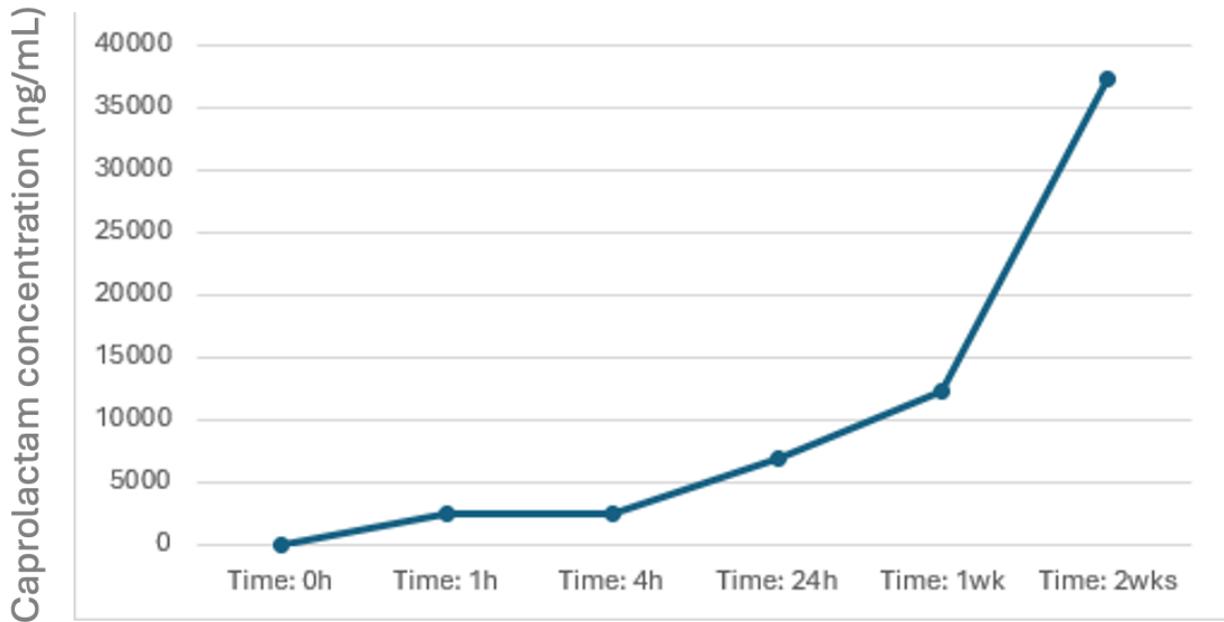
#### 4.6 Chromatography and Mass Spectrometry Analysis

All samples for aim 1 and 2 were analyzed by liquid chromatography high resolution mass spectrometry (LC-HRMS) using the the Sciex 5600 TripleTOF and the aim 3 population study was analyzed by both LC-HRMS and gas chromatography high resolution mass spectrometry (GC-HRMS), specifically quadrupole Time of Flight (qTOF) from Agilent Technologies. Both instruments allow for more comprehensive analysis since some compounds are more amenable to detection by one technology compared to the other. LC-HRMS was used

to quantify caprolactam in all patients using a calibration curve from a purified standard. The GC-qTOF was used in an untargeted setting to look at other extractables and leachables with potential health effects. The GC used was the Agilent 8890 oven couple to the 7250 Accurate-Mass q-TOF mass spectrometer. The column used was the DB-5MS UI from (30m x 0.25 x 0.25). The inlet temperature was 280°C and the transfer line temperature 325°C. The oven program is as follow: 50°C for 1 min ramp at 25°C till 170°C hold for 1 min then ramp at 15°C until 300°C hold for 10 min then ramp at 20 °C until 325°C and hold for 5 min. The MS was set to have a solvent delay of 4 min after which a standard Electron Ionization full scan collection happened. The collection range was  $45 \leq m/z \leq 1000$ . A mix of Alkanes was run along with the samples and blanks and an extractables and leachables (E&L) Standard Mix was also run as a quality check. The Alkanes established retention indices that were subsequently used in consolidating library matches in both the Agilent E&L library and the NIST library. The data in the LC-HRMS was collected using the same protocol for our clinical research routine method but only caprolactam was quantified [33]. The acquired spectra using the GC-qTOF were then searched against a library for identification of the unknown in the sample. Caprolactam was purchased as a synthesized analytical standard to analyze on our instrument to add to our library and to confirm the presence in the experimental sample. Each detected compound has a specified retention time or index (GC) on the chromatography column, a specific mass and molecular formula, and a unique fragmentation pattern (ie: mass spectrum) that is used for library matching. Using the blank subtraction feature, we identified compounds that were detected in the aligner-free saliva, that were not present in the saliva that was in contact with aligners. We also compared the relative abundance of compounds under the different conditions or timepoints.

## 5. RESULTS

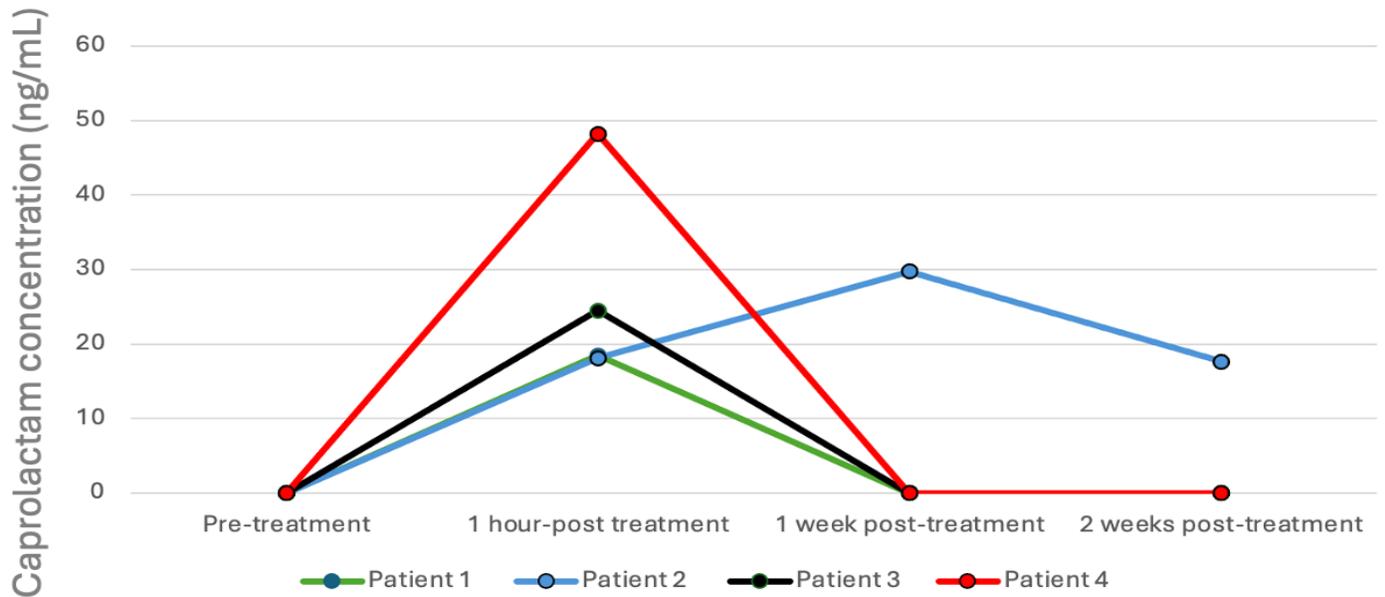
### 5.1 In-vitro Results



**Figure 5.** Caprolactam concentration at incubation time points 1h, 4h, 2h, 1wk, 2wks

The average rate of release of caprolactam is  $111.0\mu\text{g/L/h}$ . Over the entire 2-week period (336 hours), the concentration increased on average by about 111 micrograms per liter every hour. The average peak from the samples was  $37,315.45\text{ ng/mL}$  while the lowest peak was  $2400.23\text{ ng/mL}$ .

## 5.2 In-vivo Results



**Figure 6.** Caprolactam concentration of four patients followed over 2 weeks for four timepoints

Each patient peaked at 1 hour after tray delivery with 18.45 ng/mL, 18.1 ng/mL, 24.51 ng/mL, and 48.21 ng/mL caprolactam concentration. One patient increased even more to 29.74 ng/mL. All samples then decreased to either 0 or 17.64 ng/mL.

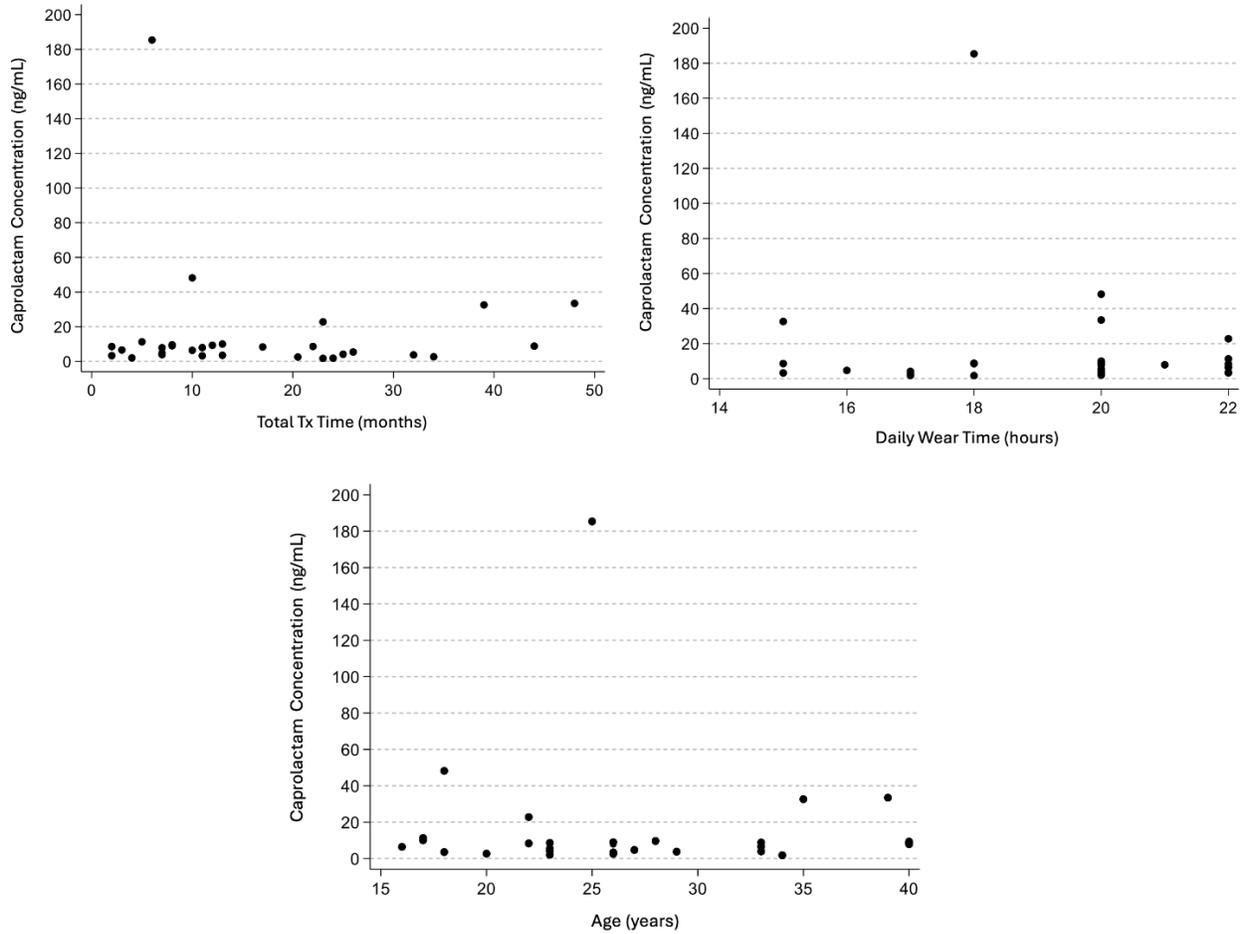
## 5.3 Population Study Results

### 5.3.1. Quantifying Caprolactam in a population of users

**Table 1.** Assessing associations between age, time since started treatment (months), daily wear time (hours), and concentration of caprolactam.

Patient	Age	TSS (months)	DWT (hours)	Concentration (ng/mL)
1	22	17	22	8.34
2	35	39	15	32.62
3	26	20.5	20	2.55
4	23	4	20	2.04
5	34	24	17	1.84
6	26	11	15	3.28
7	23	25	17	4.13
8	34	23	18	1.72
9	39	48	20	33.47
10	20	34	17	2.67
11	18	10	22	6.41
12	23	26	20	5.43
13	26	2	22	3.32
14	29	32	20	3.75
15	18	13	20	10.02
16	33	7	20	3.9
17	27	7	16	4.72
18	33	3	22	6.6
19	18	13	22	3.55

<b>Patient</b>	<b>Age</b>	<b>TSS (months)</b>	<b>DWT (hours)</b>	<b>Concentration (ng/mL)</b>
20	40	2	18	8.57
21	22	23	22	22.8
22	40	7	20	7.86
23	26	11	21	7.94
24	25	6	18	185.37
25	33	44	18	8.81
26	28	8	20	9.62
27	18	10	20	48.2
28	18	5	22	11.3
29	26	8	20	8.99
30	40	12	20	9.31
31	23	22	15	8.62



**Figure 7.** Correlation graphs between concentration of caprolactam and age, TSS, and DWT

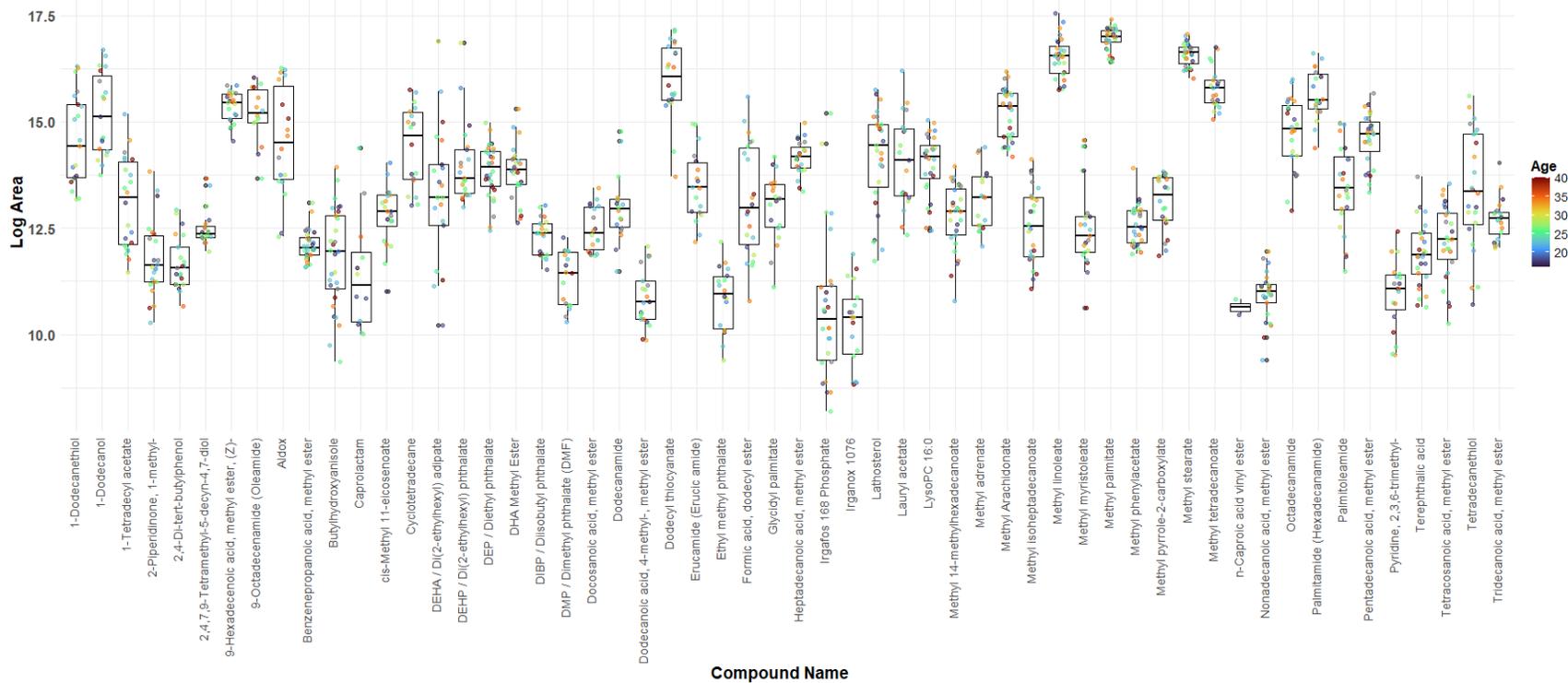
**Table 2.** Significance of spearman’s rho for concentration of caprolactam and age, TSS, and DWT

Variables	Spearman’s rho	p-value
Concentration; Age	-0.0502	0.7885
Concentration; TSS	-0.0206	0.9125
Concentration; DWT	0.1172	0.5299

### 5.3.2 Qualitative Analysis of Contaminants

Using a mass spectral library of E&L developed by Agilent Technologies for their GC-qTOF as well as the larger NIST library we have identified a variety of potentially harmful chemicals that were present in patients' saliva. E&L are chemical compounds that can be released from plastic based products with variable levels of toxicity. We will be discussing the potential significance of the effect of these compounds and their eventual correlations to other metrics in our continuation of this study.

Over 800 distinct compounds were identified with high confidence. We only kept the compounds that were present in over 60% of the population across patient saliva samples, several of which are known to have adverse health effects. A curated list of 56 compounds was shown and colored by age and time since start (Figure 8). The analysis reveals notably a variety of phthalates, amides and methyl esters known to be plasticizers and coating agents with variable health effects. Another notable observation, for a compound like methyl palmitate (a known plasticizer) seem to have low variability, compounds like caprolactam are more variable but we're also seeing some potential metabolites (n-caproic acid vinyl ester). The large array of amides is also noteworthy.



**Figure 8.** Compounds identified in more than 60% of the 31 patients in the population study

**Table 3.** Industrial Applications and Toxicological Profile of Compounds in > 60% of Samples

Compound Name	Industrial Use	Toxicological Profile	Reference
1-Dodecanethiol	Prevents premature curing in polyurethane and neoprene	Skin and eye corrosion and sensitization	[34], [35]5/5/2025 11:59:00 PM
1-Dodecanol	Used in the synthesis of biodegradable polymers and as a building block for other specialty chemicals	Low toxicity, skin and eye irritation upon prolonged exposure	[36], [37], [38]5/5/2025 11:59:00 PM
1-Tetradecyl acetate	Paints and coatings	Mild skin and eye reaction	[39]
Methyl-2-Piperidinone	Lactam, polymer synthesis intermediate	Severe skin and eye irritation. Established LD50	[40]
2,4-Di-tert-butylphenol	UV stabilizer of polymers and coatings	Serious skin and eye irritation, endocrine disruptor	[41]
2,4,7,9-Tetramethyl-5-decyn-4,7-diol	Used for multilayer polymers	Mild skin irritation and serious eye corrosion	[42]
9-Hexadecenoic acid, methyl ester, (Z)-	Lubricant, emollient, surfactant and flavor agent	Skin and eye irritation	[43]
Oleamide	Lubricant in PET processing	Skin and eye irritation, allergen	[44], [45]
Butylhydroxyanisole	Added to plastics to prevent degradation from heat and light	Cancer causing in CA	[46], [47]
Caprolactam	Building block of Nylon and variety of specialty plastics	Skin, eye, mucus and nervous toxicity	[48]
Cis-Methyl 11-elcosenoate	Lubricant and surfactant in cosmetic formulations	Mild skin and eye irritation	[49]
Cyclotetradecane	Intermediate in organic synthesis and fragrance industry	Limited data; generally considered to have low toxicity	[50]

<b>Compound Name</b>	<b>Industrial Use</b>	<b>Toxicological Profile</b>	<b>Reference</b>
DEHA/ Deha di 2 ethylhexyl adipate	Plasticizer in PVC products and food packaging materials	Potential liver effects at high doses	[51]
DEHP/di(2-ethylhexyl) phthalate	Plasticizer in flexible PVC products	Reproductive and developmental toxicity; classified as a probable human carcinogen	[52]
DEP/ diethyl phthalate	Plasticizer in personal care products and cosmetics	Potential endocrine disruptor	[53], [54]
DHA Methyl Ester	Nutritional supplement and ingredient in infant formulas	Generally recognized as safe (GRAS); high doses may cause gastrointestinal discomfort	[55]
DIBP/ Di-isobutyl phthalate	Plasticizer in plastics and resins	Reproductive toxicity; potential endocrine disruptor	[56], [57]
DMP/ Dimethyl phtalate (DMF)	Plasticizer in cellulose plastics and insect repellents	Skin and eye irritation	[58], [59]
Docosanoic acid, methyl ester	Lubricant and additive in cosmetics and personal care products	Mild skin and eye irritation	[60], [61]
Dodecanamide	Surfactant and foam stabilizer in personal care products	Low toxicity; may cause skin and eye irritation	[62]
Dodecanoic acid, 4-methyl-, methyl ester	Flavoring agent and intermediate in organic synthesis	Low toxicity; may cause mild skin and eye irritation	[63]
Dodecyl thiocyanate	Intermediate in organic synthesis and rubber industry	Limited data; may cause skin and eye irritation	[64]
Erucamide (Erucic amide)	Lubricant and slip agent in plastics and rubber	Mild skin, eye, and respiratory irritation	[44]
Ethyl methyl phthalate	Plasticizer in plastics and resins	Limited data; potential endocrine disruptor	[65]

<b>Compound Name</b>	<b>Industrial Use</b>	<b>Toxicological Profile</b>	<b>Reference</b>
Formic acid, dodecyl ester	Solvent and intermediate in organic synthesis	Limited data; may cause skin and eye irritation	[66]
Glycidyl palmitate	Reactive diluent in epoxy resins and coatings	Skin and eye irritation; potential sensitizer	[67]
Heptadecanoic acid, methyl ester	Lubricant and additive in cosmetics and personal care products	Low toxicity; may cause mild skin and eye irritation	[68]
Irgafos 168 phosphate	Antioxidant in plastics and polymers	Low toxicity; may cause skin and eye irritation	[69]
Irganox 1076	Antioxidant in plastics and polymers	Low toxicity; may cause skin and eye irritation	[70]
Lathosterol	Intermediate in cholesterol biosynthesis and biomarker in clinical studies	Low toxicity; naturally occurring compound	[71]
Lauryl acetate	Flavoring agent and fragrance ingredient	Low toxicity; may cause skin and eye irritation	[72]
LysoPC 16.0	membranes and biomarker in clinical studies	Low toxicity; naturally occurring compound	[73]
Methyl 14-methylhexadecanoate	Intermediate in organic synthesis and flavoring agent	Low toxicity; may cause skin and eye irritation	[74]
Methyl aderenate	Component in biodiesel and lubricant formulations	Skin and eye irritation	[75]
Methyl Arachidonate	Precursor in the synthesis of bioactive lipids	Inflammation and irritation in tissues upon prolonged exposure	[76]
Methyl Isoheptadecanoate	Used in formulation of fruity and floral scents in perfumes and food products	Skin or eye irritation	[68]
Methyl Linoleate	Component in biodiesel and lubricant formulations	Skin and eye irritation	[77]

<b>Compound Name</b>	<b>Industrial Use</b>	<b>Toxicological Profile</b>	<b>Reference</b>
Methyl myristoleate	Component in biodiesel and lubricant formulations	Low toxicity; may cause skin and eye irritation	[78]
Methyl palmitate	Component in biodiesel and lubricant formulations	Low toxicity; may cause skin and eye irritation	[79]
Methyl phenylacetate	Flavoring agent and fragrance ingredient	Low toxicity; may cause skin and eye irritation	[80]
Methyl pyrrole-2-carboxyate	Intermediate in organic synthesis and pharmaceutical industry	Skin and eye irritation	[81]
Methyl stearate	Component in biodiesel and lubricant formulations	Low toxicity; may cause skin and eye irritation	[82]
Methyl tetradecanoate	Component in biodiesel and lubricant formulations	Low toxicity; may cause skin and eye irritation	[83]
N-Caproic acid vinyl ester	Intermediate in organic synthesis and polymer industry	Limited data; may cause skin and eye irritation	[84]
Nonadecanoic acid, methyl ester	Lubricant and additive in cosmetics and personal care products	Low toxicity; may cause mild skin and eye irritation	[85]
Octadecanamide	Lubricant and surfactant in personal care products	Low toxicity; may cause skin and eye irritation	[86]
Palmitamide (Hexadecanamide)	Lubricant and surfactant in personal care products	Low toxicity; may cause skin and eye irritation	[87]
Palmitoleamide	Intermediate in organic synthesis and potential therapeutic	Low toxicity; may cause skin and eye irritation	[88]
Pentadecanoic acid, methyl ester	Intermediate in organic synthesis and flavoring agent	Low toxicity; may cause skin and eye irritation	[89]
2, 3, 6-trimethyl-Pyridine	Intermediate in organic synthesis, a solvent in chemical reactions, Present in low temperature coal tar and coal soot	Skin, eye, and respiratory irritation	[90]

<b>Compound Name</b>	<b>Industrial Use</b>	<b>Toxicological Profile</b>	<b>Reference</b>
Terephthalic acid	Intermediate in polyester synthesis	Low toxicity; may cause skin and eye irritation	[91]
Tetracosanoic acid, methyl ester	Lubricant and additive in cosmetics and personal care products	Low toxicity; may cause mild skin and eye irritation	[92]
Tetradecanethiol	Used in surface coatings and lubricants	May cause skin and eye irritation	[93]
Tridecanoic acid, methyl ester	Intermediate in organic synthesis and lubricant formulations	Low toxicity; may cause skin and eye irritation	[94]

## 6. DISCUSSION

Given that the recommended wear time per aligner is one week, our data demonstrating continuous leaching of caprolactam up to two weeks is noteworthy. After one week of continuous in-vitro incubation, the concentration of caprolactam reached 12.262  $\mu\text{g/mL}$ . While this value is significantly lower than the 5500  $\mu\text{g/mL}$  threshold at which chromosomal damage was observed in human lymphocytes in vitro, and much lower than the 500  $\mu\text{g/mL}$  concentration that induced mutations in mouse embryos in a spot test, it still raises questions about long-term exposure [24][95].

Further, Kelman et al. reported that human exposure to nylon-6—a polyamide made from  $\epsilon$ -caprolactam—at 0.07  $\mu\text{g/mL}$  caused eye, nose, throat, and skin irritation, albeit without systemic toxicity [96]. The caprolactam concentrations detected in saliva in our study were lower than the levels reported in the literature to cause genotoxic or irritative effects, which is reassuring. However, Triebig et al. found no chemosensory effects at a maximum exposure of just 0.005  $\mu\text{g/mL}$  over six hours, suggesting this as a potential No Observed Adverse Effect Level [97]. Ashby et al. also noted that any chromosomal damage observed was minimal and limited to chromatid gaps, further suggesting that caprolactam may have low genotoxic potential at low doses [98].

In our in-vivo aim, we consistently observed high caprolactam levels in saliva after one hour of aligner wear, followed by a decline to levels near or below the quantification limit (<2  $\text{ng/mL}$ ). The population study also revealed very low caprolactam levels, much lower than in the in-vitro and in-vivo samples. These differences may be attributed to biological processes such as detoxification or metabolic shunting in live patients, which reduce accumulation. This aligns with studies by Martina et al. and Pratsinis et al., where incubation of aligner materials influenced cytotoxicity. Martina's in-vitro study found mild cytotoxic effects with incubation,

whereas Pratsinis did not observe cytotoxicity in non-incubated conditions—highlighting the potential role of incubation and degradation in leaching [31] [16]. Moreover, the decrease in concentration over time could be influenced by dilution due to increased salivary flow or individual variability in the oral environment. It is worth noting that we are also finding a variety of compounds related to caprolactam degradation (caproic acid in two patients) and multiple amides (oleamide, palmitamide, palmitoleamide, octadecanamide) all of which are products of degradation of Nylon fibers. These can be seen in Table 3. Although this contamination could be coming from other sources, the fact that they were not found in the blanks and that they were all present in appreciable amounts and in almost all patients highly suggests that they are leaching from the aligners. It is also noteworthy that the variable amount of compounds like caprolactam could suggest it is being metabolized whereas the lower variability of compounds like methyl palmitate or methyl stearate suggests that they might be relatively more prone to accumulation in an in-vivo setting.

Interestingly, our study found no significant associations between variables such as age, duration of aligner treatment, or hours of daily wear and the concentration of caprolactam. This suggests that external factors may have limited influence on caprolactam leaching, underscoring the need to better understand the materials themselves and their biocompatibility over time.

We acknowledge several limitations in our study. One is the potential for contamination from the plastic tubing used in the mass spectrometer. Although we subtracted blank values to mitigate this, trace influences may still persist. Another limitation is that we did not control the use of lip products such as lipstick, which could interfere with salivary composition and compound detection.

An additional avenue for future research may include measuring salivary amylase levels to explore whether enzymatic activity correlates with the amount of caprolactam leached. Investigating whether the acidity of the oral cavity plays a role in caprolactam concentration may also help clarify the chemical behavior of leachates in different oral conditions. Future in-vitro and in-vivo experiments could be expanded to include longer incubation periods—such as 4 and 8 weeks—to better simulate extended wear times, particularly for patients who wear trays for longer than the standard two-week duration.

It would also be valuable to assess whether Invisalign's hypoallergenic aligners differ in their leaching profiles compared to standard versions. Moreover, while the 3M Unitek Transbond XT adhesive used for attachments does not list caprolactam as a component, it could still be worthwhile to evaluate whether the number or surface area of attachments correlates with caprolactam levels in saliva [99]. A more targeted chemical analysis using the Agilent Technologies mass spectral library of extractables and leachables may help identify other potentially relevant compounds and correlate them with patient factors or treatment variables.

It would be interesting to further assess the interaction of caprolactam with the human body, as Sun et al. found that caprolactam can form conjugates with amino acids under certain conditions [100]. It would be interesting to explore whether similar conjugates appear in patient saliva samples. Feature-based molecular network analysis could be used to investigate potential metabolic or biotransformation pathways in vivo and how these may differ between individuals.

It is also worth noting that 3D-printed aligners have been shown to release more cytotoxic compounds than thermoformed ones. This distinction highlights the importance of manufacturing methods and their implications on biocompatibility, especially given that aligners can be worn continuously for months or even years.

Finally, assessing the cytotoxicity of the measured caprolactam concentrations using gingival fibroblasts may help determine their impact on oral cell viability and membrane integrity. This would provide a more clinically relevant understanding of the biocompatibility of aligners. Given that aligners can be worn continuously for extended periods, further studies are warranted to investigate the long-term effects of low-dose exposure to caprolactam and related compounds in the oral environment.

## 7. CONCLUSION

This study demonstrates that clear aligners release detectable levels of caprolactam—both in vitro and in vivo. In vitro, concentrations increased over time when incubated at body temperature, while in vivo data revealed an initial spike in salivary caprolactam levels within one hour of aligner delivery, followed by a gradual decline over a two-week period. This pattern suggests that salivary enzymes or other physiological mechanisms may play a role in detoxifying or clearing these substances from the oral environment.

While clear aligners provide significant advantages in terms of aesthetics, comfort, and convenience, these findings raise important questions about the long-term biocompatibility of aligner materials. Although the levels we detected in our study were lower than those reported in previous toxicological literature to cause chromosomal or systemic damage, the presence of leached compounds—especially when aligners are worn for months or years—warrants further investigation. Our findings support the need for expanded research, including exploring patient variability in metabolism, and cytotoxicity assessments using relevant oral cell models.

Furthermore, this study highlights the importance of transparency in material composition and the need for more rigorous and standardized biocompatibility testing as the aligner market continues to expand. As clear aligners become increasingly popular among patients of all ages, ensuring their safety must remain a priority.

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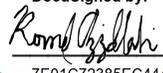
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