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STRUCTURAL PROPERTIES AND RELEASE OF BISPHENOLS DURING
ORTHODONTIC ALIGNER AGING

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ABSTRACT

The widespread use of clear aligners in orthodontic treatment has increased intraoral exposure to polymer-based devices, raising concerns about their chemical stability and biological safety. Orthodontic aligners are subjected to several stresses within the oral cavity, thereby increasing the risk of degradation and the potential release of endocrine-disrupting compounds. The current scientific literature is limited and mainly focuses on bisphenol A, neglecting the wider effects of related substances and their mixtures. Therefore, this study aimed to directly compare the chemical and biological safety of modern clear aligner materials through a combined experimental approach. Commercial polymers and 3D-printed resins underwent standardised in vitro ageing in solvents that mimicked various intraoral conditions. The resulting solutions were analysed using Fourier Transform-Infrared Spectroscopy (FT-IR) and Gas Chromatography-Mass Spectrometry (GC-MS). Additionally, extracts were examined for estrogen-like biological activity with an MCF-7 cell proliferation assay to detect effects not identified by the analytical techniques. The results showed that ageing, chemical release, and biological effects varied depending on the material. Chemical analysis did not identify specific compounds, such as bisphenol A or related chemicals. These findings were supported by biological assays, which found no estrogenic activity. This study provided a clear, clinically relevant assessment of the safety of orthodontic clear aligner materials, demonstrating that combining analytical and biological testing enhances risk assessment, supports evidence-based clinical decisions, and guides future advances in clear aligner therapy.

INTRODUCTION

The use of orthodontic clear aligners has risen considerably in recent years, driven by their aesthetic appeal, patient comfort, and advancements in polymer science (Meade et al., 2023; Meade & Weir, 2022). Despite their clinical success, these devices are characterised by prolonged intraoral exposure due to continuous contact with saliva and varying physical and chemical conditions. Clear aligners are made from multipolymer materials whose composition may include residual monomers, additives, and degradation products that can potentially be released during intraoral clinical use (Tamburrino et al., 2020).

Mechanical stress, thermal variations, and chemical ageing may further influence the stability of these materials, raising concerns about their long-term chemical and biological safety (Elkholy et al., 2019). Many studies have demonstrated that intraoral conditions, including temperature variations, pH changes, and mechanical loading, can accelerate polymer ageing and alter the overall properties of orthodontic materials (Dalaie et al., 2021; Srinivasan et al., 2024). In this context, particular attention has been given to the potential release of bisphenols and related compounds, known for their endocrine-disrupting effects (Iliadi et al., 2020; Rochester & Bolden, 2015).

However, the literature lacks a complete assessment of the chemical substances released from specific aligner materials and their potential biological effects, especially at exposure levels below the detection thresholds of standard analytical methods. While several studies have focused on the detection of Bisphenol A (BPA) using targeted analytical techniques, emerging evidence suggests that biphenolic analogues and complex mixtures may exert biological effects even when present at concentrations below conventional detection limits (Caballero-Casero et al., 2016; Yang et al., 2011).

Although different regulatory limits and standardised analytical methods are available, growing scientific evidence suggests that biological effects of polymer-derived compounds can occur at very low concentrations, sometimes below the detection limits of conventional techniques (Rosenfeld, 2018; Heindel et al., 2020). As a result, assessing the safety of aligners cannot rely solely on chemical characterisation but must involve an integrated approach that combines analytical evaluation with biological effect testing. (Ferreira et al., 2025; Iliadi et al., 2020).

A multi-level strategy enables not only the detection of potentially released substances but also the assessment of their biological activity, resulting in a more complete and clinically relevant risk evaluation. To overcome this limitation, the current research was designed to examine the chemical and biological safety of orthodontic clear aligner materials through a stepwise, integrated experimental approach.

Spectroscopic analysis using Fourier Transform Infrared Spectroscopy (FT-IR) was utilised as an initial screening method to identify characteristic functional groups and detect potential chemical-structural changes related to material ageing (Khijmatgar et al., 2022; Tamburrino et al., 2020). This was followed by Gas Chromatography–Mass Spectrometry (GC-MS) as a second-stage, targeted analytical technique to identify and characterise low-molecular-weight substances released from the materials under specified extraction conditions, consistent with previously validated experimental approaches (Kotyk & Wiltshire, 2014; Yang et al., 2011).

Finally, a cell-based bioassay using estrogen-responsive MCF-7 cells was included as a complementary biological tool to assess possible estrogen receptor–mediated effects of selected material extracts, providing functional insight into biological activity that may not be captured by chemical analysis alone (Caballero-Casero et al., 2016; Rosenfeld, 2017). Through this integrated and sequential methodological framework, the present work aims to provide a comprehensive evaluation

of the chemical and biological safety of orthodontic clear aligner materials, thereby supporting evidence-based clinical use and future material development.

BACKGROUND

Clear Aligner Therapy: Evidence-Based Biomechanics and Clinical Application

Clear aligner therapy (CAT) has become one of the most widely used treatment alternatives in modern orthodontics. Initially considered an aesthetic device, CAT has evolved into a conventional modality, supported by advances in digital workflows. CAT is increasingly replacing fixed multibracket appliances, which remain the gold standard for managing more complex cases. In contrast, clear aligners employ computer-generated trays to deliver controlled, defined, and incremental forces. Their widespread use is attributed to several patient-centred benefits, including major aesthetic requests, improved comfort, reduced mucosal irritation, and improved oral hygiene. All these advantages stem from the removable nature of clear aligners, which makes these devices popular. These advantages have contributed to a marked increase in aligner use globally over the past two decades. Recent research indicates that over 90% of orthodontists now use aligners, particularly among adults aiming for aesthetic orthodontic treatment (Meade et al., 2023; Meade & Weir, 2022). Practitioner surveys provide additional evidence for this shift, noting that aligners are perceived as comfortable, aesthetically acceptable, and more conducive to improved oral hygiene than traditional appliances (Arveda et al., 2025). This trend is determined by market expansion, increased clinician confidence in digital planning, and enhanced biomechanical performance of current systems. (Chakraborty et al., 2025)

The evolution of CAT occurred alongside advances in polymer science, technological advances, and biomechanical modelling. In fact, early aligners were composed of single-layer thermoplastics and were susceptible to stress relaxation and unstable force delivery. Following generations introduced multilayer thermoplastic technologies with improved elasticity, force retention, and resistance to deformation (Almalki et al., 2024; Caruso et al., 2024). The innovation of these materials promoted

more predictable biomechanical force systems and greater longevity during wear. Digital planning software has also advanced, allowing clinicians to previsualize staged tooth movement, manage force vectors using attachments, and engage differential-thickness zones to modulate biomechanics and force systems.

Nevertheless, mechanical limitations persist despite material advancements (AlMogbel et al., 2024; Baneshi et al., 2025). The manufacturing constraints of thermoformed plastics and the passive nature of removable appliances continue to limit the precision of force transmission, especially for complex movements such as bodily translation, torque, large rotations, or significant vertical corrections. These limitations underline the necessity for critical evaluation when incorporating CAT into comprehensive orthodontic planning. (Figure 1).



Figure 1 - Clear aligner

Various authors have compared aligners with traditional fixed braces and discovered that aligners are equally effective for mild-to-moderate dental issues (Muro et al., 2023; Robertson et al., 2020). Recent studies also indicate that aligners can reduce appointment times and emergency visits by approximately 20-30%, simplifying the clinician's workload and improving patient satisfaction (Yassir et al., 2022; Zheng et al., 2017). Additionally, there are benefits for gum health: patients using

aligners exhibit 25% less plaque and 40% less gum inflammation compared to those with braces (Miranda e Paulo et al., 2024). Nevertheless, current research reveals a significant variation in the accuracy and predictability of tooth movement (Shrivastava et al., 2023; Vigneshkumar et al., 2024). A recent study reported that the accuracy of tooth turning ranges from 36% to 65%, even with improved attachments and meticulous planning (Chakraborty et al., 2025). To improve these outcomes, clinicians may employ optimised attachments or supporting tools, such as rubber bands, small screws, or partial braces (AlMogbel et al., 2024). Controlling posterior anchorage remains another challenge. Achieving maxillary molar distalisation up to 3,5 mm is often feasible, but bodily mesialisation beyond 2,5–2,75 mm frequently causes unwanted coronal tipping or arch deformation (Baneshi et al., 2025) (D'Antò et al., 2024). Extraction therapy further tests the biomechanical limits of aligners. Cases involving the extraction of a single mandibular incisor have demonstrated favourable results with aligners when carefully selected and supported by rectangular attachments. However, in cases requiring four premolar extractions, loss of torque, bowing, and inadequate space closure are common issues. These cases often necessitate supplementary hybrid mechanics or skeletal anchorage (Benedetti et al., 2025; D'Antò et al., 2024). There is limited scientific evidence regarding the long-term stability of CAT. Preliminary studies report mild-to-good retention outcomes and high patient satisfaction; however, systematic reviews note the lack of rigorous long-term data and variability in retention protocols across clinical settings (Shrivastava et al., 2023). These limitations underscore the importance of cautious interpretation of digital treatment plans. Strategic use of overcorrection, slow staging, and auxiliaries can help mitigate predictable shortcomings.

The use of clear aligners in paediatric orthodontics has increased significantly over recent years. Advances in materials science and more accurate digital planning have contributed to this growth. Although initially proposed for adults, current evidence shows that CAT is effective in mixed dentition. This is particularly true for children aged 8–13 years. Expert consensus supports this trend: clinicians often use aligners during the second transitional dentition phase, mainly for orthodontic

rather than orthopaedic goals (Arveda et al., 2025). Dentoalveolar treatments, such as aligning teeth and expanding dental arches, are generally more achievable with aligners due to their design, which applies a gentle, consistent force suitable for tooth movement rather than bone remodelling. In growing children, skeletal changes depend more on orthopaedic interventions or growth-modification appliances, which CAT cannot provide because its force cannot influence bone structure.

Several recent research studies have evaluated the clinical performance of aligners in early treatment. Studies on paediatric systems show that CAT can achieve clinically meaningful dentoalveolar expansion during mixed dentition, with an average expansion of around 3 mm, though results vary across studies (Zheng et al., 2017). However, randomised trials consistently indicate that rapid maxillary expanders produce greater skeletal expansion than aligners, suggesting that CAT mainly results in dentoalveolar rather than skeletal changes in growing patients (Kasper-Sonnenberg et al., 2014). Additionally, aligners are effective for correcting crossbites and minor discrepancies, and digital staging features allow better control over arch form and eruption pathways (Chakraborty et al., 2025). Systems that include mandibular advancement components show promise in managing Class II malocclusions, with positive effects and higher acceptance among adolescents.

Additionally, studies report high satisfaction among paediatric patients and parents, linked to better integration into school and social environments, and improved periodontal health compared with traditional appliances (Miranda e Paulo et al., 2024; Zheng et al., 2017).

Despite these advantages, significant limitations deny the use of aligners in paediatric orthodontics. Shrivastava et al. assessed that specific movements are less predictable in children than in adults, due to eruption variability and compliance challenges (Shrivastava et al., 2023). Additionally, current evidence suffers from methodological heterogeneity, limited sample sizes, and a lack of long-term follow-up, emphasising the need for larger clinical trials to fully define the role of CAT in paediatric populations (Chakraborty et al., 2025).

Therefore, CAT cannot replace all interceptive or orthopaedic interventions; it functions as a valuable therapeutic alternative for suitably selected paediatric patients. Its aesthetic benefits, enhanced comfort, periodontal advantages, and compatibility with digital workflows make it especially attractive in modern early orthodontic practice, even though clinicians must recognise its biomechanical limitations and utilise evidence-based diagnostic and planning strategies.

To surpass these assessments, expert-based evaluations aimed to establish a clinician consensus on the correct use of CAT, such as the 2025 International Modified Delphi Study consensus by Arveda et al. The International Modified Delphi Study offers valuable insights into the real-world performance of aligners and the collective conclusions of experienced clinicians (Arveda et al., 2025). Over three rounds, the authors produced 47 consensus statements covering biomechanical capabilities, clinical indications, diagnostic requirements, retention strategies, and extra clinical factors influencing appliance selection. There was a clear consensus on the necessity of an exhaustive pre-treatment diagnosis, including panoramic radiographs, cephalometric imaging, and digital setups, to ensure accurate staging and movement prediction. The authors concluded that aligners are highly effective for tipping movements, moderately effective for small rotations and limited distalization, and minimally effective for bodily movements, vertical displacements exceeding 1.5 mm, and large rotations.

These conclusions are consistent with most of the literature and emphasise the mechanical limitations of CAT. The International Modified Delphi Study also revealed that hybrid approaches, such as combining aligners with elastics, skeletal anchorage, or sectional fixed appliances, are often necessary to achieve reliable results in complex malocclusions, including cases involving extraction therapy and severe sagittal or vertical discrepancies. Regarding patient groups, experts affirmed that aligners are effective for orthodontic objectives but not for orthopaedic ones (Chakraborty et al., 2025).

Finally, lifelong retention was strongly advised, with fixed retainers preferred in the mandibular arch and removable retainers in the maxillary arch. Apart from biomechanical considerations, the authors

identified scientific evidence and dependable customer service as the most significant factors in choosing an aligner system, while price and commercial promotion were less consistently influential. Overall, these expert opinions are consistent with current literature by highlighting CAT as highly technique-sensitive appliances whose success relies on careful case selection, meticulous planning, and thoughtful integration of auxiliary mechanics. (Table 1)

TABLE 1 - STATEMENT FOR THE CONSENSU DEFINITION BY ARVEDA ET AL. 2025

STATEMENT	Agreement %	Consensus	Round
Tipping	72	Yes	1
Mesialization Max Molars <2.75mm	75	Yes	2
Mesialization Mand Molars >2.75mm	87.5	Yes	2
Distalization Mand Molars >2.75mm	83.3	Yes	2
Distalization Max Molars >3.5mm	83.3	Yes	3
Intrusion Max Premolars/Canines >1.5mm	87.5	Yes	2
Intrusion Mand Premolars/Canines >1.5mm	87	Yes	2
Intrusion Max Molars >1.5mm	87.5	Yes	2
Intrusion Mand Molars >1.5mm	95.8	Yes	2
Intrusion Mand Incisors >1.5mm	71.7	Yes	2
Intrusion Max Incisors >2mm	70.8	Yes	3
Extrusion Max Premolars/Canines >1.5mm	73.9	Yes	2
Extrusion Mand Premolars/Canines >1.5mm	75	Yes	2
Extrusion Max Molars >1.5mm	91.7	Yes	2
Extrusion Mand Molars >1.5mm	87.5	Yes	2
Rotation Incisors <10°	75	Yes	2
Rotation Incisors 10–20°	91.7	Yes	3
Rotation Incisors >20°	79.1	Yes	3
Rotation Premolars/Canines <10°	75	Yes	3
Rotation Premolars/Canines >20°	75	Yes	3
Rotation Molars <5°	100	Yes	3
Rotation ≤1.5° Per Aligner Incisors	75	Yes	3
Rotation ≤1.5° Per Aligner Prem/Can	83	Yes	3
Rotation ≤1.5° Per Aligner Molars	75	Yes	3
Attachments Rotation	70.8	Yes	3
Attachments Torque	79.2	Yes	3
Attachments Distalization	75	Yes	3
Attachments Extrusion	75	Yes	3
Premolar Extraction Effectiveness	70.8	Yes	3
Mand Incisor Extraction	79.2	Yes	3
4 Premolar Extraction	70.8	Yes	3
Mixed Dentition Transverse Ortho Goal	76	Yes	1
Use In 8–13 Yrs	70.8	Yes	2
Orthopedic Movements Only	71.4	Yes	2
Class III Orthopedic Goal	85.7	Yes	2
Deepbite Orthopedic Goal	71.4	Yes	2
Openbite Orthopedic Goal	71.4	Yes	2
Scientific Background Influence	87	Yes	2
Customer Service Influence	75	Yes	1
Radiographs Necessity	80	Yes	1
Digital Setup Necessity	100	Yes	1
Retention Rate	100	Yes	1
Retention Duration Forever	84	Yes	1
Retention Type Mand	78.3	Yes	1
Retention Type Max	84	Yes	1

Chemical Structure and Behaviour of Thermoplastic Polymers in Clear Aligner Therapy

The clinical performance of CAT heavily depends on the chemical structure and the physical and mechanical properties of the thermoplastic polymers used in aligner fabrication. Over recent years, aligner materials have evolved from simple monolayer thermoplastics to complex multilayer systems and, more recently, to directly 3D-printed polymers. This progression mirrors the expansion of aligner indications from minor alignment to more complex orthodontic movements (Tamburrino et al., 2020; Caruso et al., 2024; Vigneshkumar et al., 2024). The development of these materials has been driven by the need to improve force delivery, reduce stress relaxation, and enhance dimensional stability under intraoral conditions (Dalaie et al., 2021; Fang et al., 2020). Since aligners rely entirely on the interaction between polymer properties and digital staging protocols, their biomechanical effectiveness is fundamentally determined by molecular composition, viscoelastic response, thermoelastic behaviour, and ageing processes within the oral cavity (Elkholy et al., 2019; Khijmatgar et al., 2022; Srinivasan et al., 2024). A thorough understanding of these material-related factors is crucial for critically evaluating the strengths and limitations of current CAT systems and for explaining discrepancies between planned and actual tooth movements in clinical practice (Arveda et al., 2025).

Polyethylene terephthalate (PET) and polyethylene terephthalate glycol-modified (PETG) are the most extensively studied thermoplastics in orthodontic research. PET is a semi-crystalline polyester with aromatic rings connected by ester bonds, providing high tensile strength, rigidity, and optical clarity. However, its high fragility limits its suitability for orthodontic appliances that undergo repeated deformation and require sustained force delivery. The addition of glycol groups in PETG disrupts crystalline regions, resulting in a more amorphous polymer with greater durability, improved formability, and reduced fracture risk during thermoforming (Fang et al., 2020; Nicita et al., 2024). These characteristics have led to the widespread adoption of PETG for clear aligners. Despite these advantages, PETG exhibits significant viscoelastic behaviour. Multiple in vitro studies have

demonstrated substantial stress relaxation, especially within the first 24 to 72 hours of simulated intraoral exposure (Dalaie et al., 2021; Khijmatgar et al., 2022). This behaviour closely relates to polymer chain mobility and water-induced plasticisation, which decrease stiffness and alter force transmission. As a result, PETG aligners are effective for simple tipping and alignment movements, but their predictability diminishes when used for complex bodily movements that require sustained force levels.

Thermoplastic polyurethane (TPU) is increasingly considered a viable alternative to PETG. As a segmented polymer composed of soft and hard domains, TPU exhibits elasticity, toughness, and abrasion resistance. Consequently, TPU-based aligners demonstrate superior shape recovery and fatigue resistance compared to PETG, providing more stable force delivery over time (Bandić et al., 2024; Migliorati et al., 2025). Comparative studies suggest that TPU aligners have lower stress-relaxation rates and greater mechanical resilience, which may be beneficial for extended wear and for movements that require consistent force.

Although PET, PETG, and TPU are the predominant thermoplastic materials for aligner fabrication in contemporary orthodontics, ethylene-vinyl acetate (EVA) and polycarbonate (PC) are still used. EVA is a copolymer mainly used in orthodontic retainers rather than active aligners. Its low elastic modulus and high flexibility provide long-term durability but limit its ability to generate effective orthodontic forces. Therefore, EVA is typically utilised for passive appliances or retention stages rather than for active tooth movement (Robertson et al., 2020; Tamburrino et al., 2020).

PC, characterised by carbonate linkages and a rigid aromatic backbone, demonstrates high impact resistance and dimensional stability. While PC offers excellent mechanical strength and transparency, its high stiffness and complex processing methods have restricted its wider use in aligner production. Research indicates that PC-based materials may be too rigid for comfortable orthodontic force application and are more likely to crack if not properly thermoformed (Fang et al., 2020).

Advances in additive manufacturing have enabled the direct production of aligners via 3D printing, eliminating the need for traditional thermoforming. These directly printed aligners are generally

produced from photopolymerizable resins, which fundamentally differ from conventional thermoplastics by forming crosslinked polymer networks instead of linear chains (Shirey et al., 2023; Tartaglia et al., 2021).

A primary advantage of 3D-printed aligners is their enhanced geometric accuracy and reproducibility. While some studies report greater accuracy for thermoformed aligners, 3D printing avoids the thickness reduction and distortion associated with heating and vacuum forming (Bichu et al., 2023; Lo et al., 2024). Furthermore, digital workflows allow precise control of aligner thickness and stiffness within a single appliance, facilitating more refined biomechanical management.

Nevertheless, the mechanical behaviour of 3D-printed aligners remains a subject of ongoing research, especially under intraoral ageing conditions. Photopolymer resins may exhibit higher initial rigidity but also increased fragility and greater sensitivity to water absorption and incomplete polymerisation (Elshazly et al., 2024; Staderini et al., 2024a)). Comparative studies indicate that 3D-printed materials can experience rapid changes in elastic modulus after simulated intraoral ageing, which may affect force consistency (Tivanani et al., 2026; Zecca et al., 2025). Moreover, factors such as printing orientation, layer thickness, post-curing protocols, and resin composition significantly influence mechanical properties, further introducing variability (Sayahpour et al., 2024).

Recent studies have explored shape-memory polymers and hybrid resin systems that react to thermal stimuli, potentially enabling dynamic force adjustment. Although these technologies show promise, current evidence is mainly *in vitro*, and their long-term clinical effectiveness remains unconfirmed (Tivanani et al., 2026). These materials are generally based on modified polyurethane formulations designed to improve force transmission through enhanced thermoelastic behaviour. Their multiphase molecular structure allows partial shape recovery at intraoral temperatures, supporting a slower, continuous release of orthodontic forces compared to traditional PETG-based aligners (Zecca et al., 2025).

The mechanical behaviour of aligner materials is described by viscoelastic principles, in which stress and strain responses depend on both time and temperature. In the oral environment, aligners undergo continuous deformation, masticatory forces, and temperature variations, all of which affect force delivery.

Thermoformed PETG aligners exhibit significant stress relaxation, with a notable reduction in force within the first days of wear (Dalaie et al., 2021; Srinivasan et al., 2024). This effect is intensified by water sorption, which acts as a plasticiser, increasing chain mobility. TPU-based aligners show lower stress relaxation and enhanced force stability, likely due to the presence of hard segments that limit molecular rearrangement (Bandić et al., 2024; Migliorati et al., 2025). Additionally, shape-memory polymer systems demonstrate reduced stress relaxation and better elastic recovery under cyclical thermal and mechanical loading, leading to greater force consistency over time. This behaviour may facilitate improved aligner tracking and biomechanical efficiency, especially during the early stages of wear. Although short-term ageing seems to have a limited impact on their mechanical response, data on long-term intraoral stability remain scarce and warrant further research. (Table 2). From a clinical perspective, these material advancements may partly explain the improved predictability observed with certain tooth movements, although they do not fully overcome the fundamental biomechanical limitations of CAT recognised by expert consensus (Arveda et al., 2025).

TABLE 2 – BIOMATERIALS PROPERTIES BY SRINIVASAN ET AL.

Material	Chemical Structure	Typical Manufacturing	Mechanical Behavior	Thermoelastic Properties	Clinical Advantages	Limitations
PET	Semi-crystalline polyester with aromatic rings and ester linkages	TF	High stiffness, high tensile strength, brittle	High Tg, limited elastic recovery	High optical clarity, dimensional stability	Excessive rigidity, fracture risk, poor fatigue resistance
PETG	Amorphous polyester with glycol-disrupted crystallinity	TF	Moderate stiffness, pronounced viscoelasticity	Lower Tg, increased flexibility	Easy thermoforming, good transparency, patient comfort	Rapid force decay, limited bodily movement control
TPU	Segmented polymer with soft and hard domains	TF/ML	High elasticity, good fatigue resistance	Broad thermoelastic range, shape recovery	Improved force consistency, resilience	Higher cost; processing sensitivity
EVA	Amorphous copolymer	TF	Very low modulus, highly flexible	Limited elastic memory	High comfort; suitable for retainers	Inadequate for active orthodontic movement
PC	Aromatic polymer with carbonate groups	TF	Very high stiffness and impact resistance	High Tg, low flexibility	Excellent strength and clarity	Excessive rigidity; poor comfort
3D-PRINTED RESINS	Cross-linked photopolymer networks	Direct printing	3D High initial stiffness; brittle behavior	Highly formulation-dependent	High geometric accuracy; digital control	Variability; aging sensitivity; limited clinical validation
SMART POLYMERS	Multiphase polyurethane-based or shape-memory systems	ML/TF	Controlled elasticity, improved force continuity	Temperature-activated shape recovery	More constant force delivery; improved tracking	Proprietary composition; limited independent data

TF(Thermoforming); ML (multilayer); Tg (glass transition temperature)

Intraoral Ageing of Clear Aligners

The ageing of clear aligners within the oral environment significantly impacts their mechanical performance and overall treatment efficiency. This is a multifactorial process resulting from the combined effects of water absorption, enzymatic activity, variations in intraoral pH, thermal cycling, and mechanical wear. All these factors induce chemical degradation and microstructural changes in aligner materials (Elkholy et al., 2019; Elshazly et al., 2024). As a result of ageing, material properties progressively change, with a notable reduction in elastic modulus. Identifying stiffness loss is therefore a critical parameter for determining optimal aligner replacement intervals in clinical settings. In vitro studies have shown that commonly used thermoplastics, such as PETG and TPU, experience decreases in elastic stiffness and optical transparency after simulated intraoral ageing. Additionally, surface roughness gradually increases, promoting plaque accumulation and biofilm formation, which pose potential risks to periodontal health (Brandão et al., 2024; Šimunović et al., 2024). Water sorption has also been shown to accelerate stress relaxation, leading to a gradual reduction in force intensity over time and potentially affecting the predictability of force delivery during orthodontic CAT (Srinivasan et al., 2024). Recent research has examined the release of micro- and nanoparticles from aligner materials resulting from wear and degradation. (Table 3). Both thermoformed and additively manufactured aligners have exhibited polymer particle release under simulated clinical conditions. However, current evidence does not establish a strong association with adverse health outcomes, and the clinical significance remains unclear (Torkomian et al., 2025; Wulff et al., 2022). Therefore, further biocompatibility and toxicological studies are required to clarify potential long-term implications. Clinically, material deterioration related to ageing may contribute to discrepancies between planned and actual tooth movements, especially in complex orthodontic cases requiring precise and stable force systems. These challenges were highlighted in the International Delphi Consensus Study by Arveda et al., which identified force

decay, material alteration, and reduced predictability of bodily tooth movements, torque expression, and vertical corrections as key limitations in CAT. (Arveda et al., 2025).

TABLE 3– INTRAORAL AGEING OF BIOMATERIALS

Material	Intraoral Ageing Behaviour
PET	Water absorption increases brittleness; transparency loss
PETG	Significant stress relaxation and force decay
TPU	Better force stability than PETG; moderate water uptake
EVA	Minimal degradation; limited force generation
PC	Risk of cracking after aging
3D-PRINTED RESINS	Water sorption; mechanical degradation; layer-dependent aging
SMART POLYMERS	Reduced stress relaxation; long-term behaviour under evaluation

Release of Substances from Thermoplastic Medical Devices

The common use of polymeric medical devices has increased scientific interest in their long-term chemical stability and biological safety. In orthodontics, clear aligners constitute a category of polymeric devices due to their prolonged intraoral use and continuous contact with oral tissues. Within the oral environment, aligners are continuously subjected to mechanical forces, temperature fluctuations, chemical exposure, and biological interactions, all of which can contribute to material degradation and the release of substances (Fang et al., 2020). The chemical structure and viscoelastic properties of thermoplastic polymers significantly influence their susceptibility to degradation processes (Srinivasan et al., 2024; Tamburrino et al., 2020). While aligner materials are generally considered biocompatible, accumulating experimental evidence indicates that chemical and mechanical ageing may promote the release of low-molecular-weight compounds. These compounds warrant further investigation due to the potential for chronic exposure and cumulative biological effects during prolonged orthodontic treatment. Degradation of polymeric medical devices results from the combined effects of chemical reactions and mechanical stress. Thermoplastic polymers commonly used in clear aligners, such as PETG and TPU, possess bonds that are vulnerable to degradation in moist environments (Lyu & Untereker, 2009). Water absorption acts as a plasticiser, enhancing polymer chain mobility and accelerating viscoelastic phenomena such as stress relaxation and creep (Bhate & Nagesh, 2024).

Mechanical loading exacerbates these degradation processes. Aligner insertion and removal, orthodontic force application, and intermittent masticatory loads repeatedly deform the material. These actions lead to surface fatigue and the formation of microcracks, increasing the surface area susceptible to chemical attack and promoting further degradation (Elshazly et al., 2024; Sayahpour et al., 2024). Manufacturing factors are also significant. Thermoforming can cause uneven thickness reduction and microstructural changes. These changes accelerate material ageing and degradation

(Bandić et al., 2024). In directly 3D-printed aligners, degradation may follow distinct pathways. Incomplete polymerisation or post-curing can leave residual monomers or oligomers. These substances may be gradually released under intraoral conditions. This suggests that mechanical integrity does not always equate to chemical inertness (Lorusso et al., 2025; Wulff et al., 2022).

The oral cavity is a dynamic environment where polymer degradation is strongly modulated by external factors. Oral pH changes in response to diet and microbial activity can accelerate hydrolytic degradation of aligner materials (Dalaie et al., 2021; Nicita et al., 2024). Repeated acidic exposure may cause progressive chain scission and gradual release of degradation products. Thermal variations also contribute to material stress. Hot beverages can temporarily raise intraoral temperatures above normal levels, leading to repeated thermal cycling and polymer fatigue, especially as materials approach their glass transition temperature (Tivanani et al., 2026). These thermal effects interact with the viscoelastic properties of thermoplastic polymers, which are intrinsically temperature dependent. Saliva acts both as a lubricant, reducing friction, and as a medium with ions, enzymes, and microorganisms that interact with polymer surfaces. Exposure to saliva alters the surface roughness and mechanical properties of aligner materials, thereby facilitating bacterial adhesion and biofilm formation (Elkholy et al., 2019; Staderini et al., 2025). Mechanical wear from mastication and parafunctional habits further damages surfaces and leads to material loss. This creates conditions for particle detachment and substance release (Koenig et al., 2022; Pratto et al., 2022).

Recent studies have investigated the micro-fragmentation of polymeric medical devices and the release of micro- and nanoparticles (Zecca et al., 2025). Both thermoformed and directly printed aligners have been shown to release polymeric fragments under simulated intraoral ageing and mechanical wear, indicating that these devices may serve as sources of microplastics in the oral cavity (Quinzi et al., 2023). Additionally, polymeric degradation can result in the release of dissolved organic compounds, including residual monomers, additives, and low-molecular-weight degradation

products such as bisphenols. Although these substances are typically detected at low concentrations, chronic, repetitive exposure associated with orthodontic treatment may lead to cumulative biological effects (Iliadi et al., 2020; Pratsinis et al., 2022). This underscores the importance of in vitro bioassay studies. Research using endocrine-sensitive cell models has demonstrated that complex mixtures of released substances can provoke measurable biological responses, even when individual compounds are present below standard analytical detection limits (Kim et al., 2024; Lorusso et al., 2025). Recent systematic reviews emphasise the need for integrated evaluation strategies that combine chemical analysis, mechanical testing, and biological assessment to comprehensively characterise the safety of aligner materials (Dantagnan et al., 2025; Iliadi et al., 2020). Overall, recent evidence suggests that substance release from polymer-based orthodontic aligners is a multifactorial process influenced by material composition, manufacturing methods, and intraoral environmental factors (Ferreira et al., 2025).

Bisphenols: BPA and Structural Analogues

Bisphenols are synthetic aromatic compounds defined by two phenolic rings connected by a central bridging group. Because of their physicochemical properties, bisphenols have been largely used in the production of plastics and resins for medical and dental applications (vom Saal & Hughes, 2005; Yang et al., 2011). In recent years, accumulating evidence has identified bisphenols, particularly bisphenol A (BPA), as endocrine-disrupting chemicals that can interfere with hormonal signalling at low exposure levels (Rochester & Bolden, 2015; Zacharewski, 1998.). The extensive use of bisphenol-containing materials has led to frequent human exposure, as demonstrated by biomonitoring studies that detect BPA and its metabolites in urine, serum, saliva, breast milk, and amniotic fluid across diverse populations (Stahlhut et al., 2009; Venisse et al., 2014). In response to BPA restrictions, various analogues have been introduced as substitutes; however, emerging evidence indicates that many of these compounds also retain endocrine activity (Bittner et al., 2014). Within this scientific background, the potential contribution of medical and orthodontic devices to cumulative bisphenol exposure has become an area of increasing scientific and clinical concern (Eliades et al., 2009; Iliadi et al., 2020).

From a chemical point of view, bisphenols share a common structure: two hydroxyphenyl groups connected by a bridge, most commonly an isopropylidene group, as in BPA. This configuration confers lipophilicity and chemical stability, supporting their use in polymeric materials such as polycarbonates and epoxy resins. BPA serves mainly as a monomer in polycarbonate plastics and epoxy coatings for food-contact materials, including water pipelines, thermal paper, and protective can linings (vom Saal & Hughes, 2005; Yang et al., 2011). The phenolic hydroxyl groups drive the biological activity of bisphenols by enabling interactions with hormone receptors and mimicking estrogenic effects. Meanwhile, the stable aromatic rings contribute to environmental persistence, resistance to biodegradation, and chronic human exposure (Rochester & Bolden, 2015). Thus, these

physicochemical properties explain both the industrial utility and toxicological relevance of bisphenols.

Among the most common structural analogues of BPA are bisphenol S (BPS) and bisphenol F (BPF). These differ mainly in their central bridging group: BPS contains a sulfonyl bridge, while BPF has a methylene bridge. Initially, these modifications were believed to reduce biological activity. However, increasing evidence indicates that these analogues retain phenolic functionalities and structural features that enable endocrine receptor interactions similar to those of BPA (Bittner et al., 2014). In addition to BPS and BPF, highly fluorinated and chlorinated analogues, such as bisphenol AF (BPAF) and bisphenol Z (BPZ), have gained attention due to their greater lipophilicity and, in some cases, higher affinity for oestrogenic receptors. Although less extensively studied, these compounds display oestrogenic and anti-androgenic effects, sometimes surpassing the potency of BPA (Rochester & Bolden, 2015). As a result, replacing BPA with structurally related analogues has raised concerns that regulatory-driven substitutions may inadvertently sustain endocrine-disrupting risks. This emphasises that the absence of BPA does not guarantee biological safety.

Bisphenols are now widely recognised as endocrine-disrupting chemicals that interfere with multiple hormonal pathways. They can function as estrogen receptor agonists or antagonists by binding to estrogen receptor alpha ($ER\alpha$) and estrogen receptor beta ($ER\beta$), as well as to membrane-associated estrogen receptors, thereby mimicking or modulating endogenous estrogen signalling (Bittner et al., 2014; Zacharewski, 1998). Over estrogenic activity, bisphenols have been shown to interact with androgen and thyroid hormone receptors, contributing to a larger spectrum of endocrine disruption (Jun Sekizawa, 2008). At the molecular level, bisphenols influence intracellular signalling cascades and induce oxidative stress, including altered DNA methylation and microRNA expression (Rosenfeld, 2017).

Reproductive toxicity is among the most consistently documented effects of bisphenol exposure. BPA exposure has been linked to disrupted steroidogenesis, altered gene expression in ovarian and testicular tissues, impaired gametogenesis, and morphological changes in reproductive organs (Pulgar et al., 2000; Ulutaş et al., 2011). In females, exposure is associated with altered follicular development and oocyte quality, while in males, it is linked to reduced sperm parameters and testicular tissue alterations (Purushothaman et al., 2015; Rochester & Bolden, 2015). Notably, co-exposure to multiple bisphenols may produce additive or synergistic endocrine effects, reflecting realistic environmental exposure scenarios (Caballero-Casero et al., 2016). Bisphenols are also implicated in metabolic dysregulation, cardiovascular changes, and neurodevelopmental effects. Epidemiological and experimental studies indicate associations with insulin resistance, obesity, and altered blood pressure regulation, particularly following early-life exposure (Rosenfeld, 2017; Stahlhut et al., 2009; Wang et al., 2020).

Within the European Union, BPA has been progressively restricted, including bans on baby bottles and limitations on food-contact materials, following assessments by the European Food Safety Authority (Heindel et al., 2020; vom Saal & Hughes, 2005). Internationally, regulatory approaches differ considerably. While some countries have implemented safety measures specifically targeting BPA, many BPA analogues remain largely unregulated despite increasing toxicological evidence. This regulatory gap has led to the widespread substitution of BPA with structurally similar compounds whose safety profiles are not yet fully characterised (Rochester & Bolden, 2015).

State of the Art on the Risk of Ingestion of Bioactive Substances from Orthodontic Devices

The potential for the ingestion of molecules released from orthodontic devices has attracted increasing scientific attention, particularly given the widespread use of polymer-based materials in modern orthodontics. Resin-based adhesives, composite cements, polycarbonate brackets, and thermoplastic aligners have all been identified as potential sources of chemical release under intraoral conditions (Eliades et al., 2009; Schuster et al., 2004). The oral environment, characterised by variable pH levels, temperature changes, salivary enzymes, and mechanical wear, promotes material degradation and facilitates the leaching of low-molecular-weight compounds and additives (Hallmann and Gerngroß, 2021). Experimental studies consistently demonstrate that substance release is highest during the initial period following appliance placement, particularly within the first 24–48 hours, and is followed by sustained, low-level leaching over time (Eliades et al., 2009). For clear aligners, prolonged daily wear (often exceeding 20 hours per day) and repeated replacement cycles may result in chronic, cumulative exposure, even when absolute release quantities remain below established toxicological limits. Biomonitoring data support the plausibility of systemic exposure, as changes in urinary and salivary concentrations of polymer-related compounds have been observed during orthodontic treatment (Iliadi et al., 2020; Zacharewski, 1998).

The current literature on chemical release from orthodontic materials has predominantly focused on BPA, often treating it as a single-marker compound to assess biological risk. This narrow approach has largely overlooked a broader group of structurally related bisphenols. Focusing solely on BPA limits the ability to capture the full spectrum of endocrine-active substances that may be released from modern orthodontic materials (Zacharewski, 1998). Consequently, the biological significance of observed release profiles may be underestimated, especially when complex chemical mixtures are present. A significant limitation in current literature is the lack of data on BPA analogues, both in general toxicological studies and specifically regarding orthodontic aligners. Structural analogues

such as BPS, BPF, BPAF, and BP have been increasingly used as BPA substitutes. However, experimental evidence in dental contexts indicates that many of these compounds retain estrogenic activity comparable to, or even exceeding, that of BPA (Umar Wazir, 2009). Despite these findings, orthodontic research has not adequately incorporated this broader toxicological knowledge into studies of aligner materials. Most existing studies have examined brackets, adhesives, or older polymeric devices, with limited relevance to contemporary multilayer thermoplastics and directly 3D-printed resins used in modern CAT (Eliades et al., 2009; Hallmann L and Gerngroß, 2021). As a result, the contribution of BPA analogues to cumulative exposure during CAT remains poorly characterised. Furthermore, many studies focus exclusively on short-term release, neglecting the prolonged and repetitive exposure typical of orthodontic treatment (Schuster et al., 2004).

Clinically relevant ageing factors such as thermal cycling, mechanical wear, repeated insertion and removal, and salivary enzymatic activity are often inconsistently included in experimental designs, despite their known effects on polymer degradation and substance release. Another major limitation is the frequent reliance on targeted chemical analyses, with limited use of effect-based biological assays capable of detecting estrogenic or endocrine-disrupting activity from complex mixtures (Caballero-Casero et al., 2016; Zacharewski, 1998). These weaknesses in the literature complicate comparisons across investigations. Therefore, the present research project addresses these limitations by implementing a multifaceted experimental framework that incorporates these ageing factors and utilises both chemical and biological assessments, thereby enabling a more comprehensive evaluation of aligner safety.

RATIONALE OF THE STUDY

CAT represents one of the most important novelties in contemporary orthodontics and is widely used in adult, adolescent, and paediatric populations. Aligners' clinical application involves prolonged exposure to polymers used in medical devices, which are worn for many hours per day.

Clear aligners are composed of advanced thermoplastic polymers that operate within the biologically complex environment of the oral cavity, characterised by pH changes, temperature variations, saliva, and repeated mechanical loading. The combined action of these factors may promote material ageing, chemical degradation, including microstructural alterations of the polymers, potentially leading to the release of molecules with biological activity (Fang et al., 2020; Srinivasan et al., 2024; Tamburrino et al., 2020). Among these substances, there are bisphenols, a class of well-recognised chemicals with endocrine activity. Recent evidence regarding their presence and biological activity in relation to orthodontic aligners remains limited and partially inconsistent. Moreover, existing studies have primarily focused on BPA, while data on structural analogues and on the overall estrogenic activity of aligner materials remain scarce (Eliades et al., 2009; Iliadi et al., 2020). These knowledge shortcomings constitute the main scientific rationale for the present study.

Therefore, based on the available evidence and the limitations identified in the literature, the present study was designed to test the biocompatibility of clear aligners and evaluate the nature and the biological activity of released molecules.

To perform this evaluation, this dissertation presents a research study that adopts an integrated experimental approach to evaluate the safety of materials used in orthodontic aligners. Structural spectroscopy is performed to identify chemical modifications of polymers associated with ageing and degradation processes. At the same time, mass spectrometry is used to find and characterise the release of bisphenols and structurally related compounds. Finally, an *in vitro* bioassay using MCF-7

cells is used to evaluate the potential estrogen-like activity of material extracts, including effects arising from complex mixtures or compounds below analytical detection limits.

The innovation of this project is the adoption of an integrated and multilevel strategy to overcome the limitations of previous studies on orthodontic aligners. Instead of focusing exclusively on BPA, the study combines research on similar structures, providing a structural, chemical, and biological analysis within a single experimental workflow. From a clinical perspective, this study promotes a more comprehensive evaluation of the biological safety of orthodontic clear aligners. Improved understanding of release mechanisms and probable estrogenic activity may support evidence-based clinical decision-making.

MATERIALS AND METHODS

This experimental research project aimed to evaluate the structural properties of orthodontic aligner materials and their potential release of biphenolic compounds under simulated ageing conditions. A multi-analytical system was used to characterise reference compounds and commercial polymeric materials. Bisphenols and a methacrylate monomer were used to validate spectral assignments and analytical sensitivity. A range of thermoplastic and 3D-printed orthodontic materials was tested after controlled in vitro ageing in solvents of differing polarity. Detailed materials, sample preparation, and analytical protocols are described below.

Tested Analytical Standards

To test the analytical instrumentation's sensitivity and provide reference compounds for detecting biomolecules in solution, a set of standard molecules was selected. These molecules, all from the bisphenol family, were selected based on their evidence and relevance in scientific literature.

Bisphenol A (BPA)

BPA is an aromatic compound composed of two phenolic halves linked by an isopropylidene bridge and is extensively employed in the production of polycarbonate plastics and epoxy resins. Its infrared spectrum is characterised by well-defined absorptions associated with phenolic O–H stretching, aromatic C–H stretching, and ring skeletal vibrations. (Figure 2).

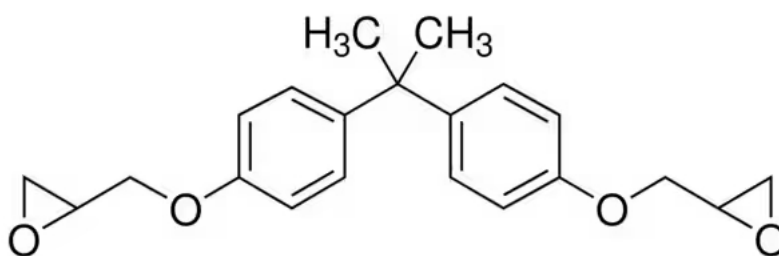


Figure 2 - Molecular structure of BPA

Bisphenol AP (BPAP)

BPAP is a structural analogue of BPA. Changes to the central bridging group alter its molecular geometry and vibrational response. (Figure 3).

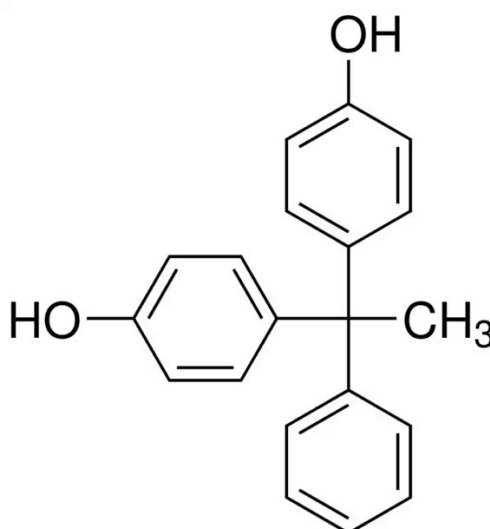


Figure 3 - Molecular structure of BPAP

The reference spectrum used for Fourier-transform Infrared (FT-IR) spectroscopy shows differences in band positions and relative intensities compared to BPA, especially in the fingerprint region. These features helped evaluate FT-IR's ability to resolve small structural differences in the bisphenol family. (Figures 4 and 5).

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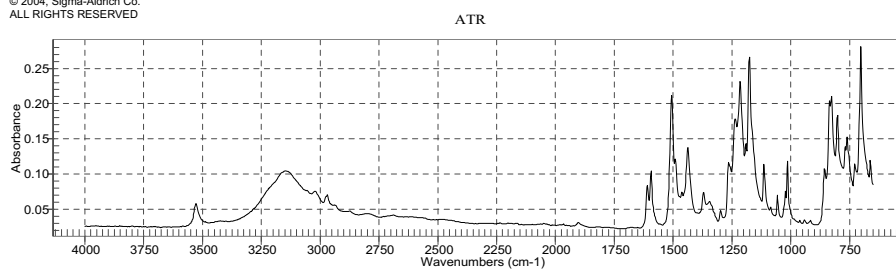


Figure 4 - Reference ATR Spectrum of BPAP

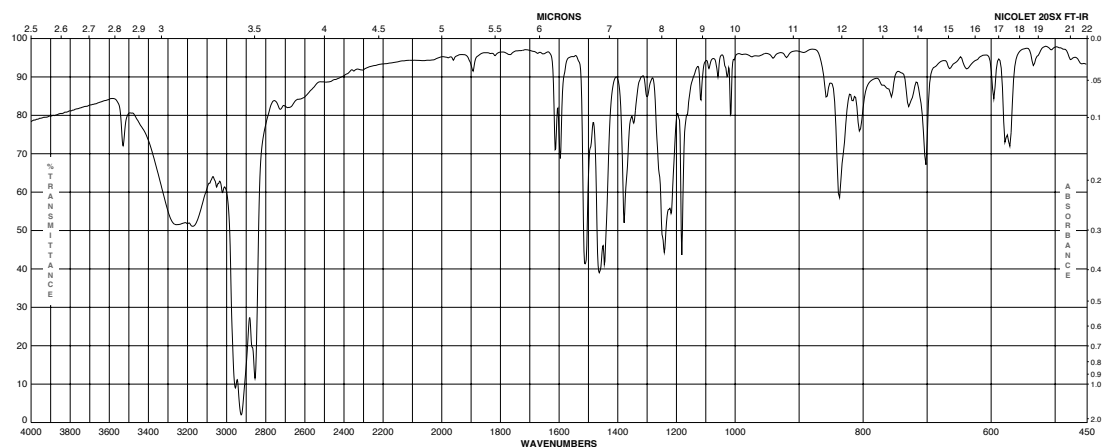


Figure 5 - Reference FT-IR Spectrum of BPAP

Bisphenol E (BPE)

BPE consists of two phenolic rings connected by an ethylene bridge. It is often used as an analytical standard in spectroscopic studies. (Figure 6).

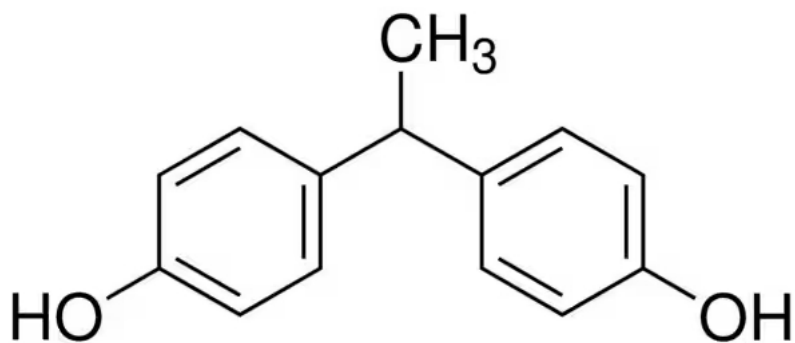


Figure 6 - Molecular structure of BPE

Its infrared spectrum shows absorptions from phenolic O–H stretching, aromatic skeletal modes, and C–H deformation vibrations. The FT-IR reference spectrum was used to compare with spectra of other biphenolic derivatives and to confirm vibrational band assignments.

Bisphenol F (BPF)

Bisphenol F consists of two phenolic units linked by a methylene bridge. This results in a less sterically hindered molecular framework. (Figure 7).

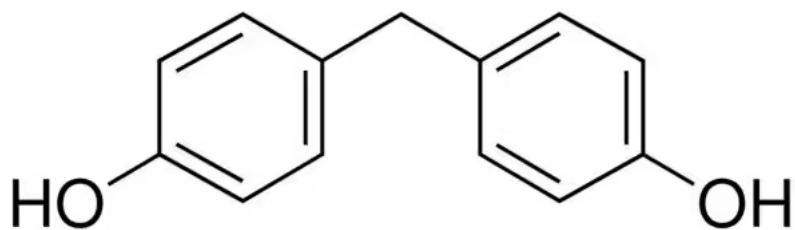


Figure 7 - Molecular structure of BPF

This structure leads to distinct absorption patterns. The FT-IR reference spectrum of BPF was used to test the protocol's sensitivity to variations in the biphenolic backbone.

Bisphenol S (BPS)

Bisphenol S features a sulfonyl group linking two phenolic rings. This increases polarity and thermal stability compared to other bisphenol derivatives. (Figure 8).

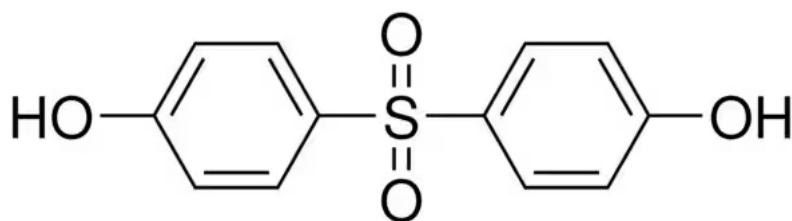


Figure 8 - Molecular structure of BPS

The sulfone group gives rise to distinct vibrational bands in the mid-infrared region. These are clear in the reference spectrum and were used to check FT-IR's sensitivity toward strongly electron-withdrawing groups.

Diurethane Dimethacrylate (DUDA)

DUDA is a bifunctional methacrylate monomer widely employed as a crosslinking agent in polymer chemistry and advanced composite materials. Its molecular structure includes urethane linkages and methacrylate groups, giving rise to characteristic absorptions in the carbonyl, N-H, and C-O stretching regions. (Figure 9).

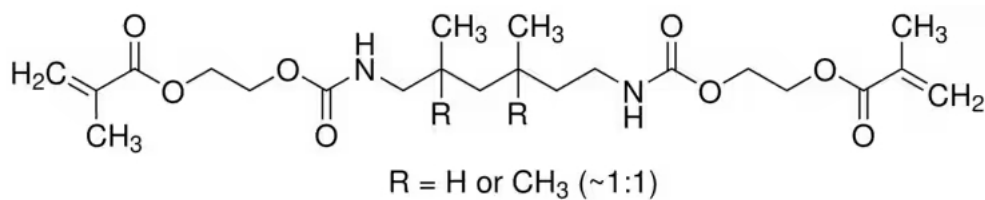


Figure 9 - Molecular structure of DUDA

The reference ATR spectrum broadened the chemical scope of the study beyond bisphenolic systems. It also validated the FT-IR method for polymer-related monomers. (Figure 10).

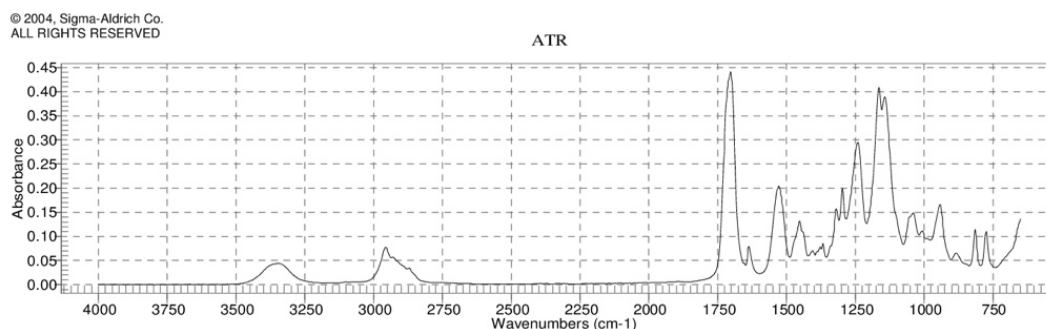


Figure 10 - Reference ATR Spectrum of DUDA

Tested Polymeric and printed materials

Once the analytical standards were established, thermoplastic materials and 3D printed resins for extraction assays were selected

Graphy®

Graphy resin TC-85 is a photopolymerizable material specifically developed for the direct 3D printing of orthodontic aligners and customised dental devices. Chemically, it is composed of a cross-linked polymer network formed through light-induced polymerisation of monomers, resulting in a close reticulated structure after curing. Graphy resin exhibits high surface resolution, but its mechanical behaviour is strongly dependent on the degree of polymerisation. Post-curing procedures significantly improve mechanical stability and reduce residual monomers, which are otherwise associated with altered mechanical performance and increased extractable content (Manoukakis et al., 2024). Clinically, Graphy resin is increasingly used for directly printed aligners and custom-made

appliances, although long-term intraoral performance and ageing behaviour remain incompletely characterised. (Figure 11).



Figure 11 - Graphy resin

Biolon®

Biolon discs are made from PETG, an amorphous copolyester formed by glycol modification of PET. PETG retains aromatic ester groups, providing dimensional stability and greater chain mobility than PET (Goracci et al., 2023). These materials have moderate elastic modulus, good impact resistance, and high clarity, making them optimal for aesthetic orthodontics. Due to their viscoelastic nature, stress relaxation and force decay occur over time, especially in wet and warm environments (Ravuri et al., 2024). In vitro studies report high biocompatibility, including high cell viability and no cytotoxicity (Kolenc et al., 2025). Clinically, Biolon discs are commonly used for thermoformed clear aligners, retainers, and bleaching trays in aligner therapy.

Erkoloc Pro®

Erkoloc Pro discs are multilayer thermoplastics based on a PETG matrix and an elastomeric component. They are engineered for flexibility and comfort. The material combines PETG's stability and transparency with the capacity of elastomers, making it suited for removable orthodontic and dental appliances. Mechanically, Erkoloc Pro has lower rigidity and greater elasticity than rigid thermoplastics, which helps distribute stress and improve comfort during long-term wear. It shows high cytocompatibility and low biological reactivity when processed and tested by standard protocols (Kolenc et al., 2025). Erkoloc Pro is used mainly for bite splints and protective appliances, especially when patient comfort is more important than orthodontic force. Its main limitations are mechanical ageing and viscoelastic behaviour, not chemical stability or biological safety. (Figure 12).

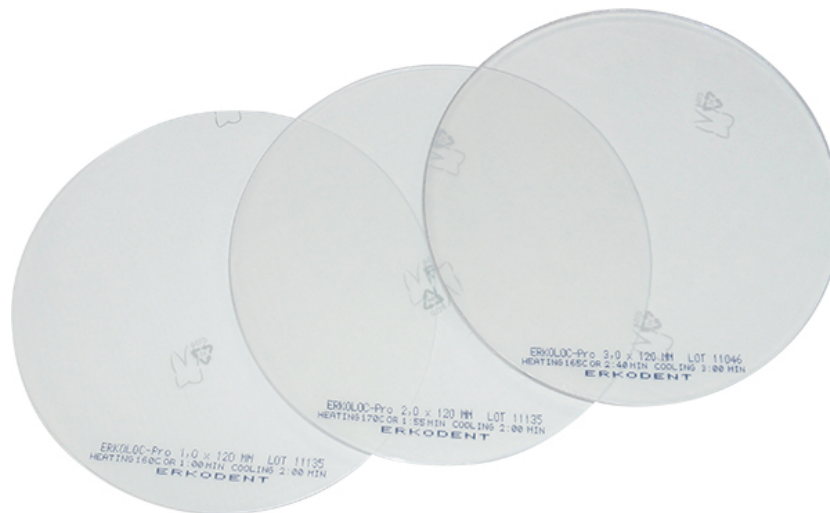


Figure 12 - Erkoloc-pro discs

Bleach

Bleach splint discs are thermoplastic sheets primarily used for whitening trays and protective oral appliances and are commonly manufactured from EVA. Chemically, EVA is an amorphous copolymer of ethylene and vinyl acetate, resulting in enhanced flexibility and a low elastic modulus. Physically, EVA-based materials show high elasticity and comfort, whereas PETG-based variants offer greater shape retention and transparency. These properties make bleach splint discs suitable for appliances requiring prolonged mucosal contact rather than active orthodontic force delivery. In vitro studies report minimal cytotoxicity and satisfactory biocompatibility of EVA when properly processed (Manoukakis et al., 2024). Although not intended for active tooth movement, these materials are relevant comparators due to their everyday clinical use and compositional similarity to the thermoplastic aligner.

3D Printing Resin – Post-Cured

Post-cured 3D printing resins, called 'Termofotopol' in experiments, are highly cross-linked photopolymers. They are made by light polymerisation, followed by thermal or photo post-curing. These multifunctional monomers are used to create dense polymer matrices with high stability (Kolenc et al., 2025). Proper post-curing improves mechanical performance and reduces residual monomers. Studies show that fully post-cured resins are cytocompatible, with high cell viability in standardised in vitro tests (Kolenc et al., 2025). These results emphasise the need for optimised post-processing to ensure safe use in mouths.

3D Printing Resin – Non-Post-Cured

Non-post-cured resins, called during the experimental phase “Formlabs”, are partially photopolymeric materials polymerised immediately after printing and are characterised by a higher proportion of unreacted monomers and oligomers. This chemical state results in reduced mechanical integrity and increased susceptibility to leaching. Experimental evidence shows that insufficient curing is associated with lower cell viability and increased extractable content, highlighting the

critical role of post-curing procedures in achieving acceptable biological performance (Goracci et al., 2023). Therefore, insufficient post-processing negatively affects biological response. These materials are included in the present study to assess the influence of polymerisation degree on material safety.

I.C.M. Essential 75

I.C.M. Essential 75 is a proprietary thermoplastic material used for the fabrication of orthodontic aligners. Due to the proprietary nature of the formulation, the exact polymeric classification cannot be determined. The material is described as an elastomeric thermoplastic material designed to combine flexibility, resilience, and sustained force delivery during aligner wear. Chemically, it appears as an elastomeric polymer chain with phase-separated domains that allow reversible deformation and shape recovery. Physically, the material exhibits a moderate elastic modulus, good fatigue resistance, and high capacity to adjust to intraoral anatomy, which supports patient comfort during all-day wear. Available in vitro data show low cytotoxicity and good cellular tolerance when properly processed (Ravuri et al., 2024). Clinically, I.C.M. Essential 75 is used in removable aligner systems as an alternative to more rigid thermoplastics. (Figure 13).



Figure 13 - ICM Essential 75® detail

Thermoforming Process and Application to the Investigated Materials

Thermoforming is a widely used industrial process for producing orthodontic aligners. In orthodontics, thermoforming is the standard method for adapting flat thermoplastic sheets to patient-specific dental anatomy, enabling the production of customised aligners and splints (Delgado et al., 2025). (Figure 14).

During thermoforming, the thermoplastic sheet is heated over its glass transition temperature and shaped over a dental model using vacuum and/or positive pressure. After cooling, the material retains the imposed geometry without chemical modification, while polymer chains undergo physical rearrangement that may affect thickness distribution, surface morphology, and mechanical and viscoelastic properties (Staderini et al., 2024). This process is well established for thermoplastic materials and combines formability with mechanical properties suitable for intraoral applications (Ravuri et al., 2024). Nevertheless, experimental studies have demonstrated that thermoforming itself may induce material alterations, including non-uniform thinning and changes in surface and mechanical characteristics, which can influence subsequent intraoral performance and ageing behaviour (Dalaie et al., 2021; Staderini et al., 2024).

In the present study, thermoforming was applied exclusively to materials supplied in disc form and specifically designed for pressure forming, including I.C.M. Essential 75, Biolon, Erkoloc-pro®, and Bleach® splint materials. These materials were selected based on their established clinical use and documented suitability for thermoforming in orthodontic and dental applications (Delgado et al., 2025). In contrast, specimens produced from 3D-printable resins were not subjected to thermoforming, as their final geometry was obtained directly through additive manufacturing followed by post-curing.

All thermoformable materials were processed using a standardised pressure-thermoforming protocol at 6 bar to minimise process-related variability among material groups. Thermoforming was performed using positive pressure within the temperature ranges recommended by the respective

manufacturers, using the same thermoforming equipment and general processing conditions for all samples. This process allowed the evaluation of material behaviours under conditions representative of routine laboratory fabrication of orthodontic appliances, while assuring methodological consistency among the experimental design (Ravuri et al., 2024).



Figure 14 - Thermoforming machine

Sample Preparation and Standardisation for Solvent Extraction

To ensure methodological rigour and comparability across all investigated materials, specimen preparation was carried out following a strict standardisation protocol. Since the release of extractable substances from polymeric materials is heavily influenced by the exposed surface area, particular attention was paid to controlling the surface-to-volume ratio during solvent extraction. All polymeric discs included in the study had the same nominal thickness; therefore, standardisation mainly focused on the planar dimensions of the samples. For this reason, a custom-cutting mould was specially made to produce samples with identical dimensions ($4\text{ mm} \times 4\text{ mm}$) from each polymeric disc and each orthodontic aligner. This method guaranteed that all samples had an equivalent surface area in contact with the extraction media, specifically ethanol (EtOH) and sodium chloride (NaCl) solution. The same dimensional specifications were strictly applied to specimens made from 3D-printable resins, ensuring consistent exposure conditions across disc-based, aligner-derived, and additively manufactured materials. This standardised specimen geometry was adopted to reduce variability caused by differential solvent contact and to facilitate direct comparison of chemical and biological results among all material groups (Figure 15).

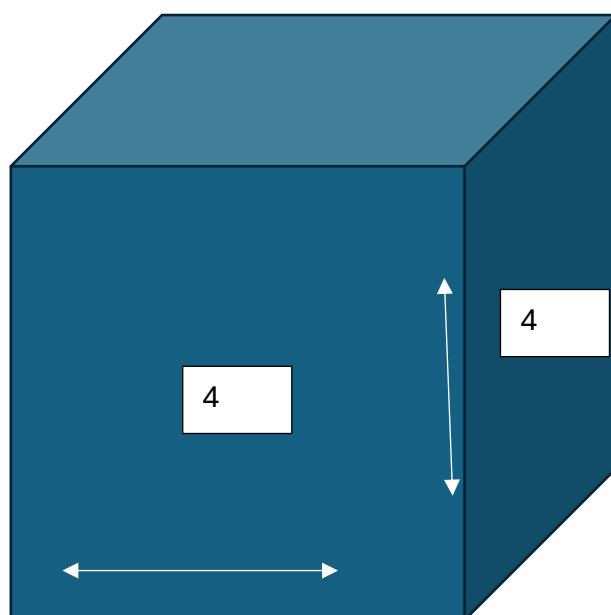


Figure 15 - Schematic representation of 4 x 4 mm mould

Ageing Protocols and Extraction Solvents

To investigate the potential release of chemical species from orthodontic materials under simulated ageing conditions, extraction protocols were designed using solvents with different physicochemical properties. Ethanol (EtOH) and sodium chloride (NaCl) aqueous solutions were selected as complementary extraction media to get a larger spectrum of extractable compounds, in accordance with established methodological approaches (Yang et al., 2011).

Ethanol, due to its intermediate polarity and organic character, is commonly used as a high-extraction-capacity medium for low-molecular-weight organic constituents and additives, and it is often considered representative of a worst-case exposure scenario. (Figure 16).

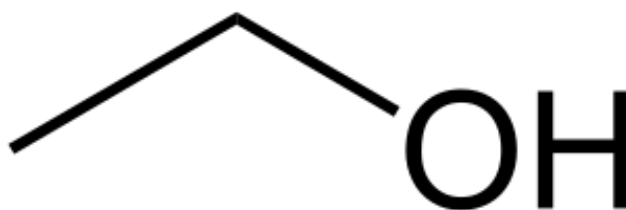


Figure 16 - Ethanol

In contrast, a NaCl solution provides a predominantly aqueous environment that more closely resembles physiological conditions, such as those of the oral cavity. (Figure 17).



Figure 17 - NaCl solution

The combined use of EtOH and NaCl, therefore, enables a comparative evaluation of material–solvent interactions under chemically distinct conditions.

The ageing and extraction protocol adopted in this study was inspired by the work of Yang et al., which serves as a methodological key reference for assessing chemical release from polymeric materials using solvents of different polarities (Yang et al., 2011). That study assessed that solvent polarity clearly influences both the qualitative and quantitative profiles of released substances, supporting the use of a multi-solvent strategy when evaluating the potential release of biologically active compounds. Therefore, the main elements of the protocol described by Yang et al., including preliminary samples sterilisation, incubation at physiological temperature, and defined mass-to-volume extraction ratios, were adopted as the conceptual basis for the present experimental study. Where necessary, these parameters were critically adapted to account for the specific geometry and dimensions of the orthodontic materials investigated.

Solutions preparation

Before incubation, all polymeric specimens were sterilised by exposing them to ultraviolet light for 30 minutes at approximately 60 cm. Throughout the experiment, to prevent microbial contamination, sample management was performed under sterile conditions in a laminar flow hood. After the sterilisation, samples were immersed in the two sterile extraction media: EtOH and NaCl solution. Incubation was performed at 37 °C for 15 days; this temperature was selected to simulate physiological thermal conditions and was consistent with the reference protocol (Yang et al., 2011). Initial mass-to-volume ratios were set according to Yang et al: 1 g of material per 1.5 mL of NaCl solution and 1 g of material per 1 mL of ethanol. During a preliminary experimental phase, these volumes did not ensure complete sample immersion; therefore, solvent volumes were increased until complete coverage was achieved. This update preserved the conceptual basis of the reference mass/volume ratios while improving the reliability of the extraction conditions. Following ageing and extraction, the resulting solutions were subjected to integrated analytical and biological evaluation. During the wait between experimental phases, the solutions were stored at -80 degrees, ready to proceed with the following experimental steps.

Fourier-transform infrared spectroscopy (FT-IR) was used to evaluate potential chemical and structural modifications of the materials associated with ageing and solvent exposure. Gas chromatography–mass spectrometry (GC-MS) was then used to detect and identify extractable organic compounds. Later, the biological activity of the extracts was evaluated using an in vitro estrogenic-responsive MCF-7 cell proliferation assay, enabling assessment of the overall estrogenic potential of the released substances. Together, these complementary approaches provided a multilayered evaluation of material behaviour, integrating structural, chemical, and functional endpoints.

Fourier Transform Infrared (FT-IR) spectroscopy

Fourier Transform Infrared (FT-IR) spectroscopy was used as a complementary analytical technique to investigate the possible presence of bisphenols and structurally related compounds in extraction solutions obtained from orthodontic polymeric materials. FT-IR spectroscopy is based on the interaction of infrared radiation with molecular vibrational modes. It provides qualitative information on chemical functional groups and molecular structure through characteristic absorption bands. Citi? Owing to its non-destructive nature and suitability for analysing both solids and liquids, FT-IR is a well-established method for screening polymer-derived compounds and low-molecular-weight organic species in complex matrices. Infrared absorption occurs when a vibrational mode is associated with a change in the molecular dipole moment. The position and intensity of absorption bands are influenced by bond strength, atomic masses, and the local chemical environment, resulting in spectra that are highly characteristic of individual compounds. In the mid-infrared region, fundamental vibrational transitions dominate and provide the primary basis for compound identification and comparison.

All spectra were acquired using Fourier Transform Infrared spectroscopy, which enables the simultaneous collection of all spectral frequencies through interferometric detection. The interferogram generated by the optical system was mathematically transformed into a spectrum expressed as absorbance versus wavenumber. This approach provides high spectral accuracy, improved signal-to-noise ratio, and excellent reproducibility, making FT-IR particularly suitable for comparative analysis of reference compounds and experimental samples (Atkins, 2014). FT-IR analyses were performed using a Bruker Vertex 70v spectrometer, operated either under ambient conditions or under vacuum to minimise atmospheric interferences arising from water vapour and carbon dioxide. Two complementary sampling modalities were employed: attenuated total reflection

(ATR) for the analysis of reference compounds and transmission mode for the study of liquid extraction samples.

ATR measurements were conducted using a diamond crystal, selected for its high refractive index, chemical inertness, and mechanical robustness. In ATR mode, infrared radiation undergoes internal reflection within the crystal, generating an evanescent field that penetrates only a few micrometres into the sample in contact with the crystal surface. This configuration allows the acquisition of spectra without prior sample preparation and is particularly suitable for solid materials and viscous compounds. (Derrick, 1999).

Transmission measurements were performed using a liquid cell with two ZnSe windows separated by a 3 μm Mylar spacer, providing a well-defined optical path length. ZnSe windows were chosen for their high transparency in the mid-infrared region and their minimal contribution to the recorded spectra. This configuration enabled the reliable acquisition of transmission spectra from liquid samples while minimising the risk of signal saturation (Derrick, 1999). (Figures 18 and 19).

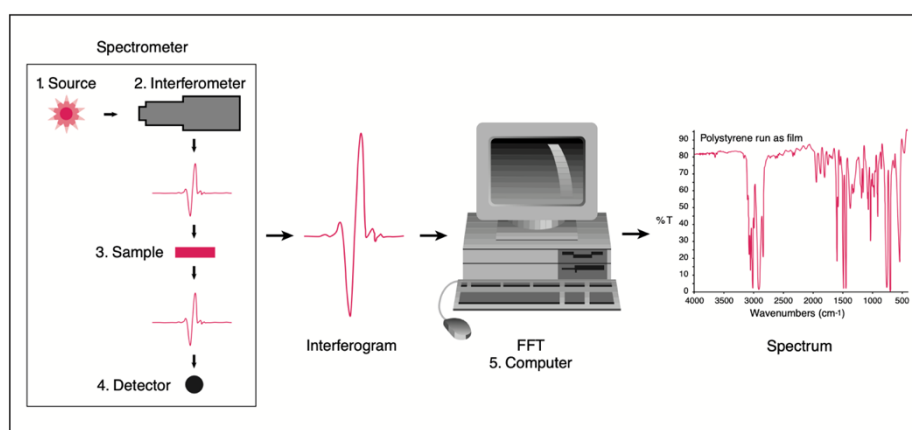


Figure 18 - The Sample Analysis Process

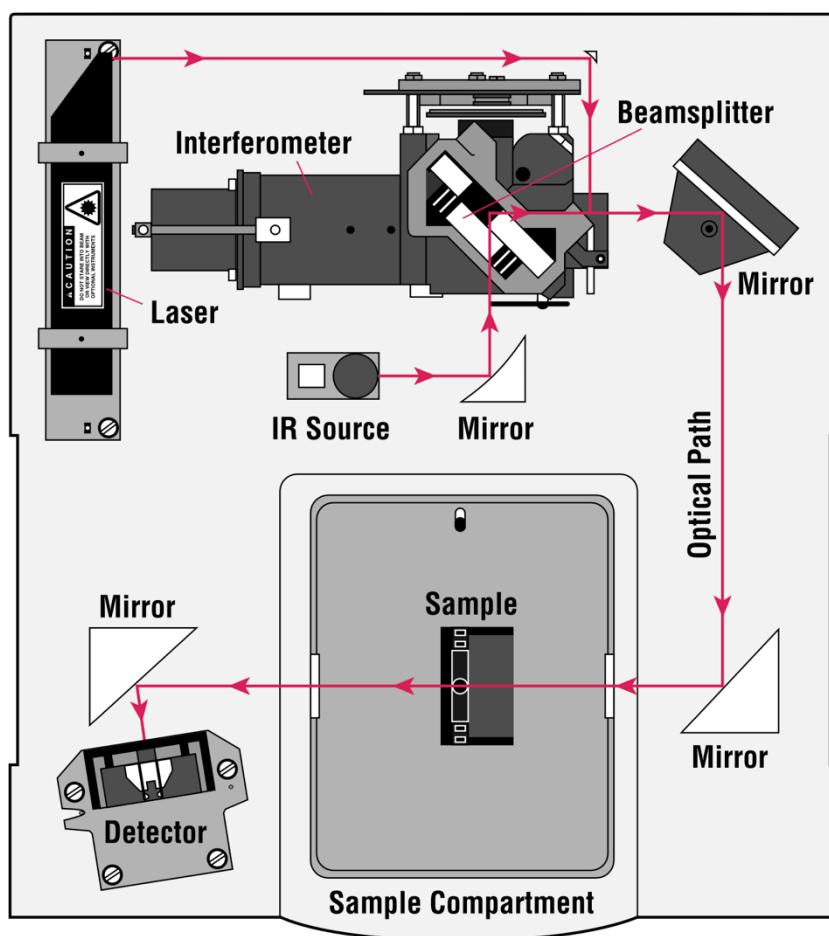


Figure 19 - A Simple Spectrometer Layout

Sample preparation and data acquisition

After incubation for 15 days at 37 °C, 200 μ L of each extraction solution was withdrawn and analysed by FT-IR. Reference compounds, including bisphenol A and all its derivatives, were investigated in ATR mode to establish their characteristic vibrational fingerprints. Liquid samples obtained from polymer extraction experiments in ethanol and physiological saline solution were analysed in transmission mode. Background spectra were acquired using pure ethanol and pure saline solution, respectively, to account for solvent and cell contributions.

For all measurements, spectra were obtained by co-adding 128 individual scans at a spectral resolution of 4 cm^{-1} . This acquisition strategy was selected to enhance the signal-to-noise ratio while maintaining sufficient spectral resolution for the identification of diagnostic vibrational features.

Spectral pre-processing included baseline correction, absorbance normalisation, and ATR correction algorithms, where applicable, ensuring consistency and comparability across different sampling modes. As a complementary proof-of-principle assessment of the analytical sensitivity and interpretative framework, an additional measurement was performed on a binary mixture containing 10% acetone in ethanol. This experiment verified the ability of the adopted FT-IR protocol to resolve and identify distinct chemical species within a solvent matrix.

Spectral analysis and interpretative criteria

Spectral interpretation was conducted with reference to the principal diagnostic regions of the mid-infrared spectrum, commonly employed for the analysis of organic and polymeric materials. Particular attention was devoted to the O–H and N–H stretching region (3600–3200 cm^{-1}), the C–H stretching region (3100–2800 cm^{-1}), the carbonyl stretching region (1750–1600 cm^{-1}), and the fingerprint region (1200–700 cm^{-1}), which contains a high density of structurally informative absorption bands. Compound identification and comparison were based on qualitative evaluation of band positions, relative intensities, and overall spectral profiles. Spectra of experimental samples were systematically compared with those of reference compounds to assess the presence or absence of bisphenol-related vibrational features. No quantitative calibration or concentration determination was performed using FT-IR spectroscopy.

Gas chromatography coupled with mass spectrometry (GC–MS)

Gas chromatography coupled with mass spectrometry (GC–MS) was employed for the investigation of bisphenols and structurally related compounds potentially released from orthodontic polymeric materials. GC–MS was selected for its high chromatographic resolving power and for the structural information provided by electron ionisation mass spectra, which is particularly advantageous for the identification of trace-level organic compounds in complex matrices. (Atkins, 2014)

Chromatographic separation was achieved on a capillary column using an inert carrier gas under temperature-programmed conditions. Analyte retention was governed by differential partitioning between the mobile and stationary phases. At the same time, separation efficiency depended on diffusion phenomena and mass-transfer kinetics, as described by classical chromatographic theory. Upon elution, analytes were transferred to the mass spectrometer and ionised. Mass spectra were acquired as ion abundance versus mass-to-charge ratio (m/z) and used for compound identification through comparison with reference standards and spectral libraries (NIST Mass Spectral Library, 2020).

A schematic representation of the GC–MS system employed in this study is reported in Figure 20.

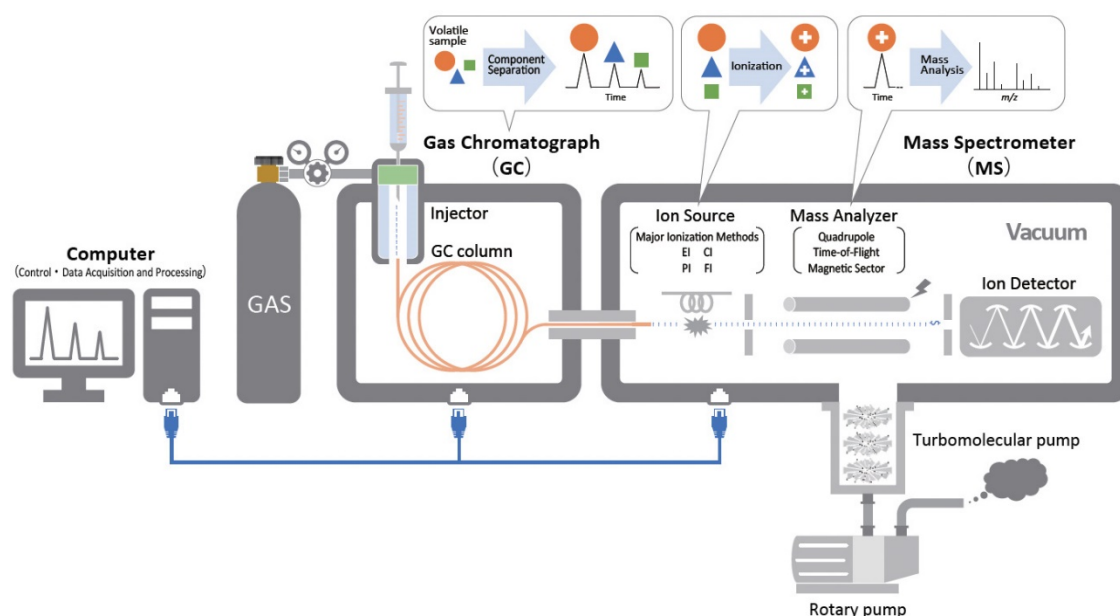


Figure 20 - GC-MS system

Native bisphenols exhibit limited volatility and thermal stability, which restricts their direct analysis by gas chromatography. To overcome these limitations, a derivatisation step was introduced before GC-MS analysis. Acetylation of phenolic hydroxyl groups was performed according to the method proposed by Kotyk and Wiltshire (Koty & Wiltshire, 2014), yielding diacetylated derivatives with enhanced volatility and improved chromatographic behaviour. The acetylation reaction converts phenolic groups into acetate esters, yielding derivatives more suitable for GC separation and EI mass spectrometric detection. This derivatisation approach has been widely applied in studies addressing bisphenol release from polymeric and biomedical materials. ref

Sample preparation and data acquisition

Following incubation, the extraction solutions obtained from the polymeric discs were evaporated to complete dryness using a centrifugal evaporator. Drying was performed under reduced pressure and mild temperature conditions to ensure complete solvent removal while minimising the risk of thermal degradation or loss of semi-volatile analytes. The resulting homogeneous dry residue provided a

consistent starting matrix for derivatisation. Derivatisation was carried out by adding 1 mL of potassium carbonate (K_2CO_3) solution to each dried sample to establish a moderately basic environment and promote deprotonation of the phenolic hydroxyl groups. Acetic anhydride was then added as a derivatising agent in three sequential aliquots (200 μ L, 200 μ L, and 100 μ L). This stepwise addition allowed controlled reaction kinetics and reduced the likelihood of incomplete acetylation or side reactions. The reaction was allowed to proceed for approximately 10 minutes at room temperature.

After derivatisation, liquid–liquid extraction was conducted by adding 200 μ L of iso-octane to each reaction mixture. Iso-octane was chosen for its low polarity, chemical inertness, and compatibility with hydrophobic acetylated derivatives, producing an organic phase suitable for direct GC–MS injection (Atkins, 2014). The organic layer was carefully collected to minimise volatilisation and contamination. An aliquot of 2 μ L of the iso-octane extract was injected into the GC–MS system for analysis under consistent instrumental conditions for all samples.

To assess the reliability of the analytical workflow, a validation experiment was conducted using BPA-spiked samples. In these experiments, bisphenol A was added before the drying step to evaluate the entire procedure, including evaporation, derivatisation, extraction, and instrumental analysis.

Samples extracted in physiological saline solution and ethanol were processed as such. At the same time, parallel aliquots were fortified with 8 μ L of a BPA solution (10 mg/mL in methanol), corresponding to an added amount of 80 μ g of BPA. Spiked and non-spiked samples were subsequently treated under identical experimental conditions. The spiked samples were used exclusively for method verification and were not included in the evaluation of bisphenol release from polymeric materials. Compound identification was based on a combined evaluation of chromatographic retention times, retention indices, and EI mass spectral fragmentation patterns. Assignments were confirmed by comparison with reference standards and spectral library data. This validated analytical procedure was subsequently applied to the experimental samples obtained from polymer incubation, and the corresponding results are presented in the following section.

MCF-7 Cell Line Assay

To evaluate the potential estrogen-like biological activity of solutions obtained from incubating polymeric materials, a cell-based proliferation assay was performed using the MCF-7 human breast cancer cell line (Liao et al., 2014). This cell line expresses a functional estrogen receptor alpha. It exhibits a well-characterised proliferative response to estrogenic stimulation, making it a widely accepted in vitro model for assessing estrogen receptor-mediated biological effects. The use of a cellular assay complements analytical techniques by providing a functional readout of biological activity. It allows the detection of receptor-mediated effects that may occur at concentrations not readily quantifiable by chemical analysis alone. MCF-7 cells were maintained under standard cell culture conditions at 37 °C in a humidified atmosphere containing 5 % CO₂. Cells were cultured in appropriate growth medium supplemented according to established protocols to ensure optimal viability and reproducibility of the proliferative response (Liao et al., 2014). For proliferation experiments, cells were seeded into multiwall plates at controlled, reproducible densities to ensure uniform growth across experimental conditions. Before exposure to test solutions, cells underwent a stabilisation period in hormone-depleted medium or in serum-free conditions to minimise basal estrogenic stimulation and enhance the assay's sensitivity to estrogen receptor-mediated effects. (Figure 21).

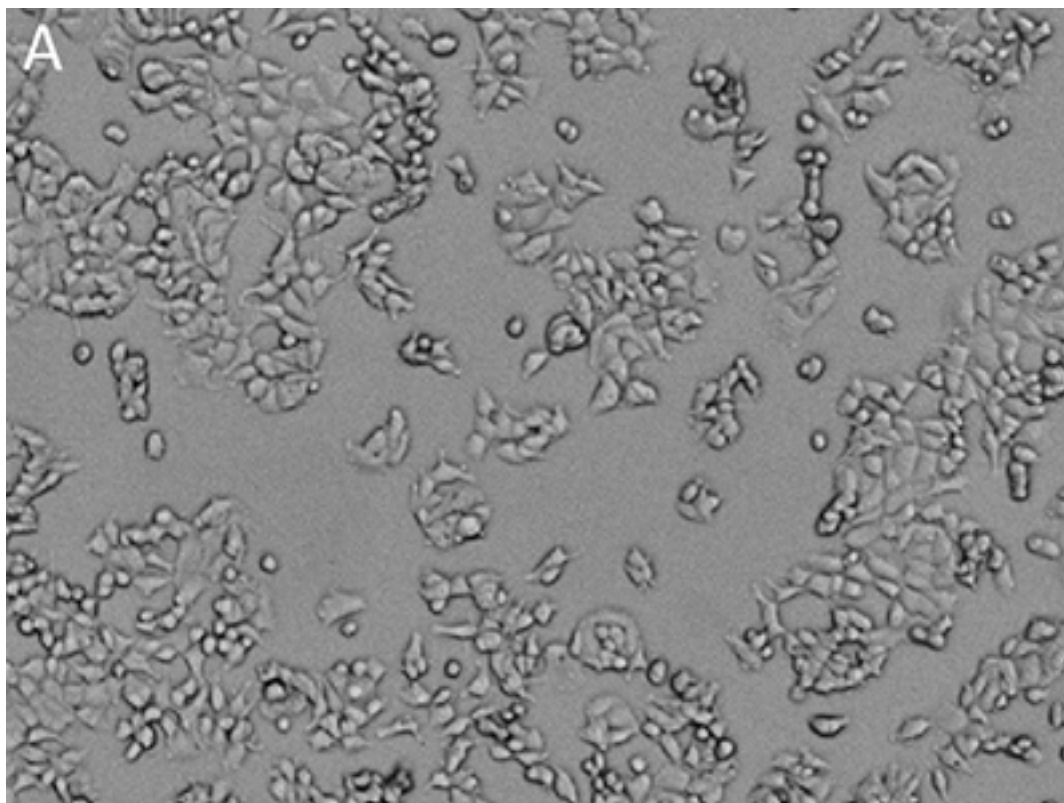


Figure 21 - MCF-7

Sample preparation and data acquisition

The solutions tested in the MCF-7 assay were obtained by incubating polymeric materials in physiological saline solution or ethanol for 15 days at 37 °C, following the same protocol used for the analytical investigations described previously. This maintains methodological consistency between chemical and biological assessments. Before exposing cells, the extraction solutions were appropriately diluted in culture medium to preserve cell viability and to prevent non-specific cytotoxic effects that could confound the interpretation of proliferation data. To ensure the reliability and interpretability of the assay, both positive and negative controls were included in each experimental series. Positive controls comprised 17 β -estradiol and selected pure bisphenols, which are well-established oestrogen receptor agonists commonly used as reference compounds in oestrogenicity assays. These controls verified the responsiveness and functional integrity of the MCF-

7 model. Negative controls consisted of the solvents used for sample preparation, administered under the same experimental conditions as the test samples. This approach allowed discrimination between effects caused by the polymer-derived extracts and those arising from the solvent matrices themselves. Cell proliferation was quantified using a colorimetric metabolic assay widely employed for evaluating in vitro cell viability and growth. After incubation with the chromogenic reagent, the product generated by metabolically active cells was measured spectrophotometrically, providing a signal proportional to the number of viable cells. Each experimental condition was assessed in multiple independent replicates to ensure statistical robustness. (Villalobos et al., 1995) Proliferation values were normalised to the corresponding negative controls, which served as the baseline for cellular growth. This normalisation process enabled consistent comparison of proliferative responses across different conditions. All experiments were performed under identical conditions to ensure comparability. Data were expressed relative to control conditions and checked for consistency across replicates. The MCF-7 assay was employed solely as a qualitative and semi-quantitative screening tool to assess potential oestrogen receptor-mediated proliferative effects and was not intended for mechanistic or quantitative dose-response analysis.

RESULTS

FT-IR Results

FT-IR spectra were recorded for a series of reference compounds and liquid samples prepared by extracting orthodontic polymer materials in ethanol and saline solutions. The reference compounds (including BPA, BPAP, BPE, BPF, BPS, and DUDA) were analysed using ATR, while the liquid extracts were examined in transmission mode, with pure ethanol and saline as background spectra. The spectra of the extraction solvents displayed absorption features consistent with their chemical compositions. Both ethanol and saline showed a broad O–H stretching band between 3600 and 3200 cm^{-1} , due to hydrogen-bonded hydroxyl groups. For ethanol, additional weak features appeared in the 1100–1000 cm^{-1} range, corresponding to C–O stretching vibrations typical of aliphatic alcohols. These solvent-related features formed the baseline spectral framework against which the spectra of the extracted samples were compared.

In contrast, the spectra of the reference compounds displayed well-defined, structured absorption patterns. Prominent bands related to aromatic ring vibrations, phenolic C–O stretching modes, and, in the case of bisphenol S, characteristic sulfonyl group absorptions were clearly visible. Each reference compound exhibited a unique vibrational fingerprint, providing a reliable basis for comparison with the spectra of the experimental samples (Figures 22-27).

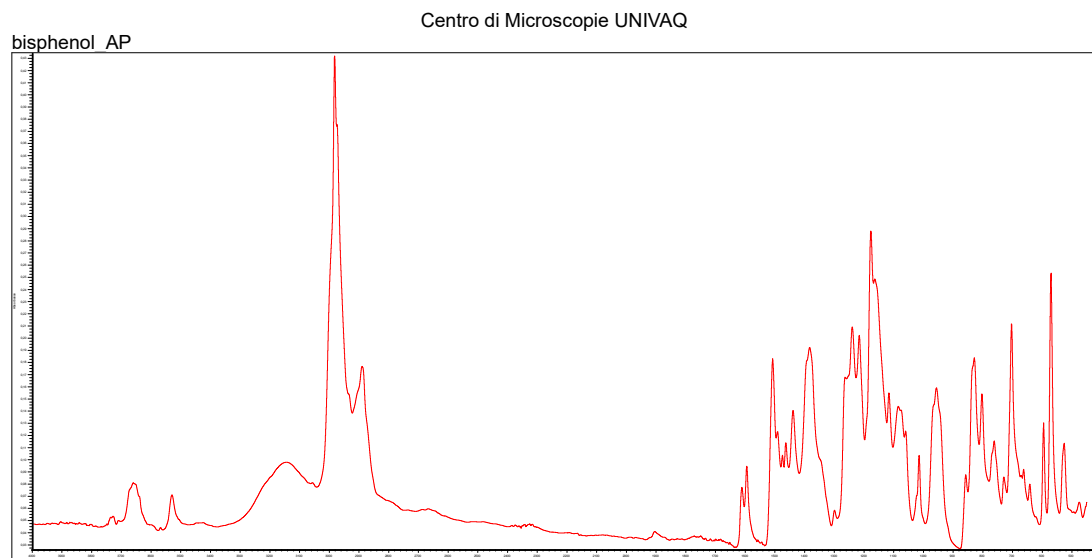


Figure 22 - BPAP Spectra

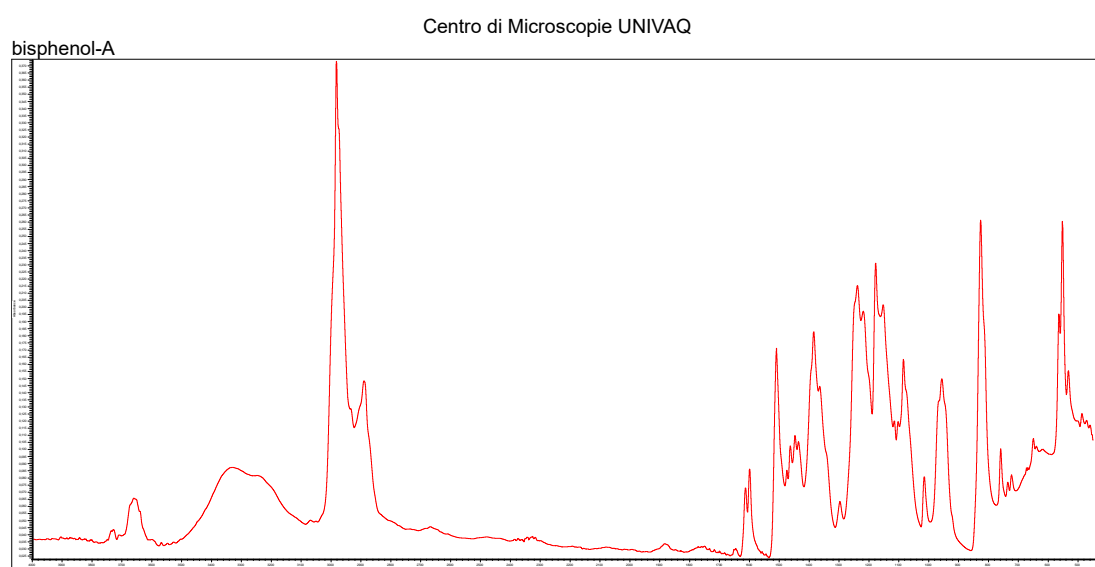


Figure 23 - BPA Spectra

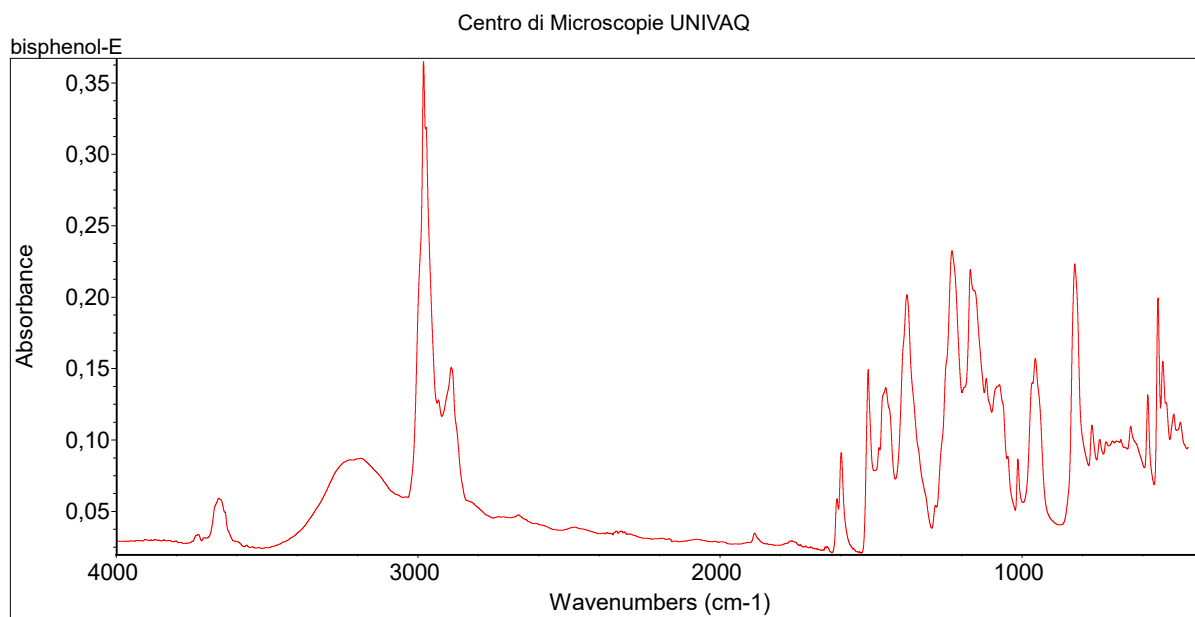


Figure 24 - BPE Spectra

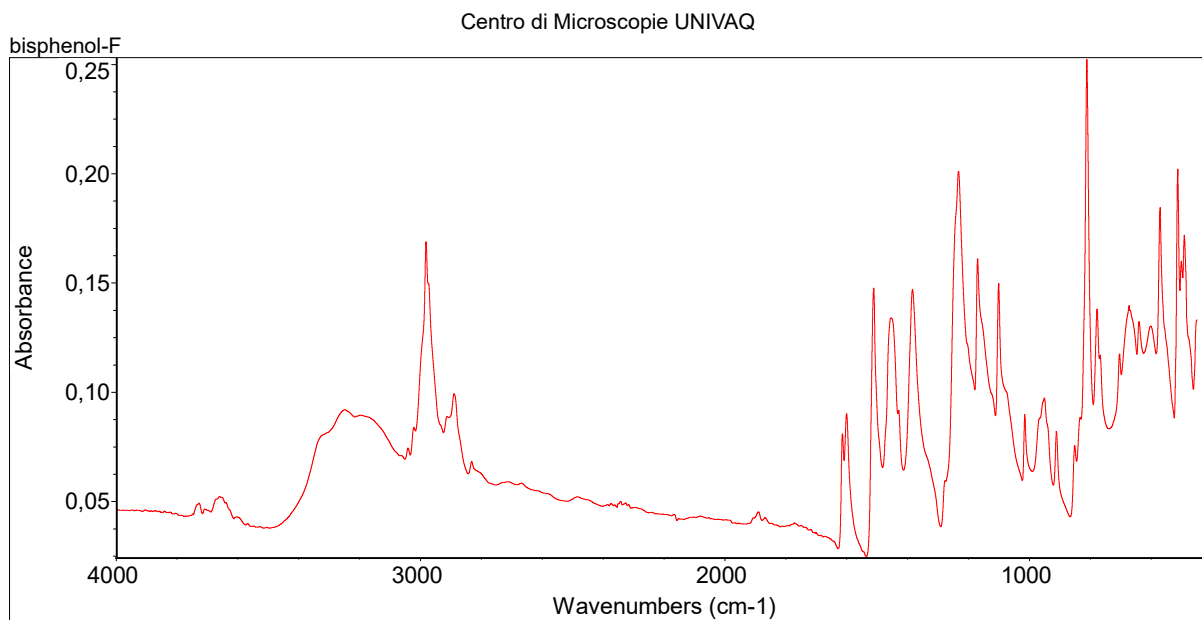


Figure 25 - BPF Spectra

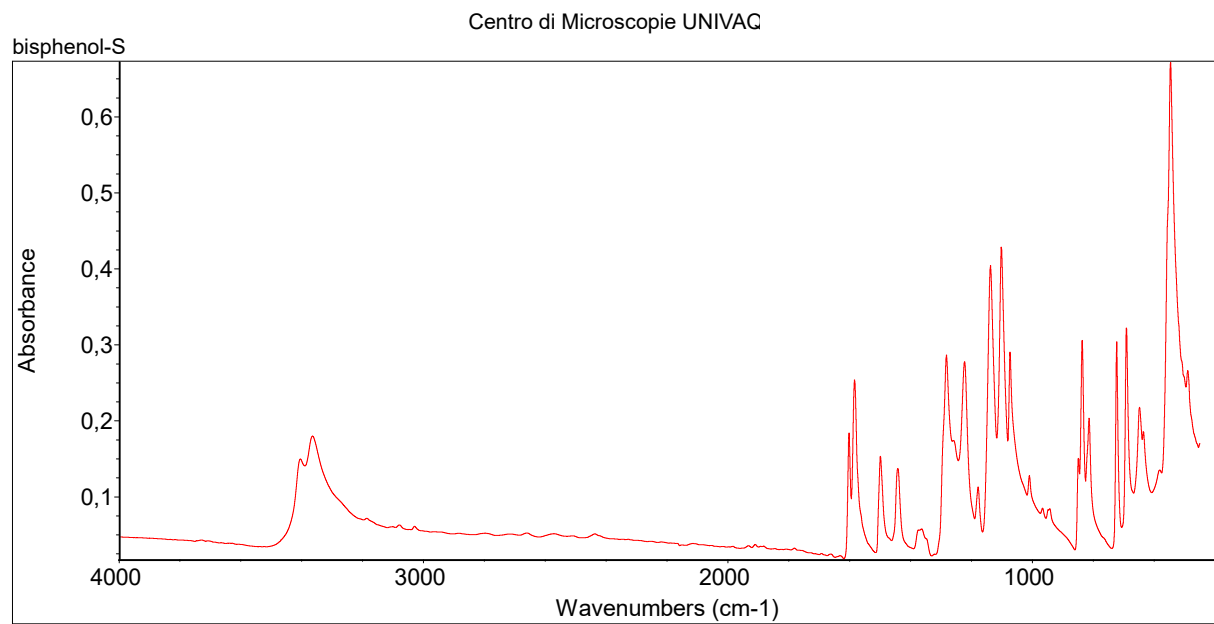


Figure 26 - BPS Spectra

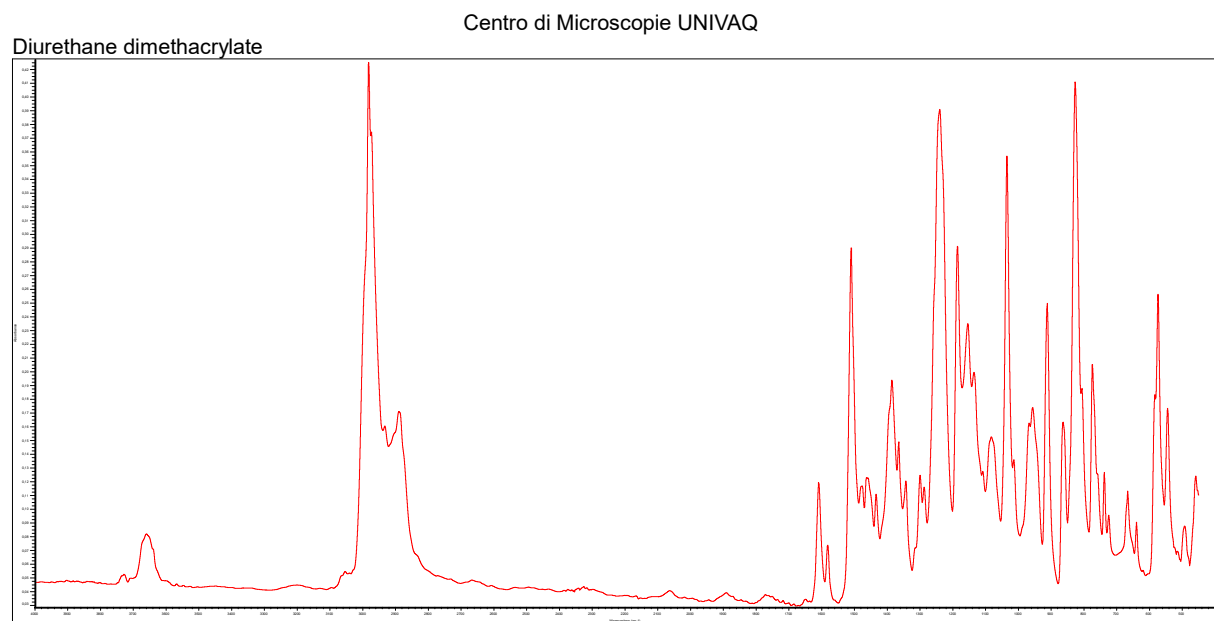


Figure 27 - DUDA Spectra

Analysis of extracted samples: absence of bisphenol-related bands

FT-IR analysis of samples extracted in both ethanol and physiological saline solution did not show absorption bands attributable to bisphenols within the method's detection limit. In particular, the characteristic aromatic ring vibrations, usually observed in the 1600–1500 cm^{-1} region, as well as the structured features in the 1300–900 cm^{-1} region seen in the spectra of the reference compounds, were not present in any of the extracted samples.

A direct comparison between the spectra of the extracted samples and those of the reference standards, conducted through spectral overlay and targeted inspection of diagnostic regions, did not show any match compatible with the presence of bisphenol A or related analogues. These findings suggest that, under the experimental conditions used, there was no FT-IR-detectable release of bisphenols from the examined polymeric materials, regardless of the extraction medium (Figures 28 and 28).

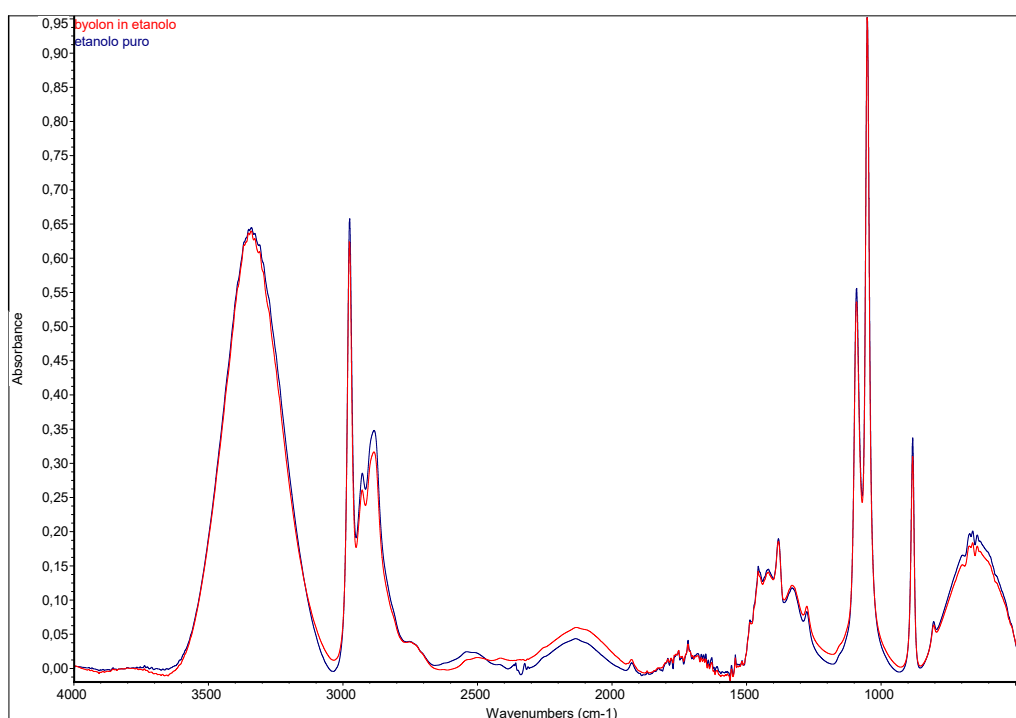


Figure 28 - Comparison between FT-IR spectra of ethanol (blue) and reference standard in ethanol (red).

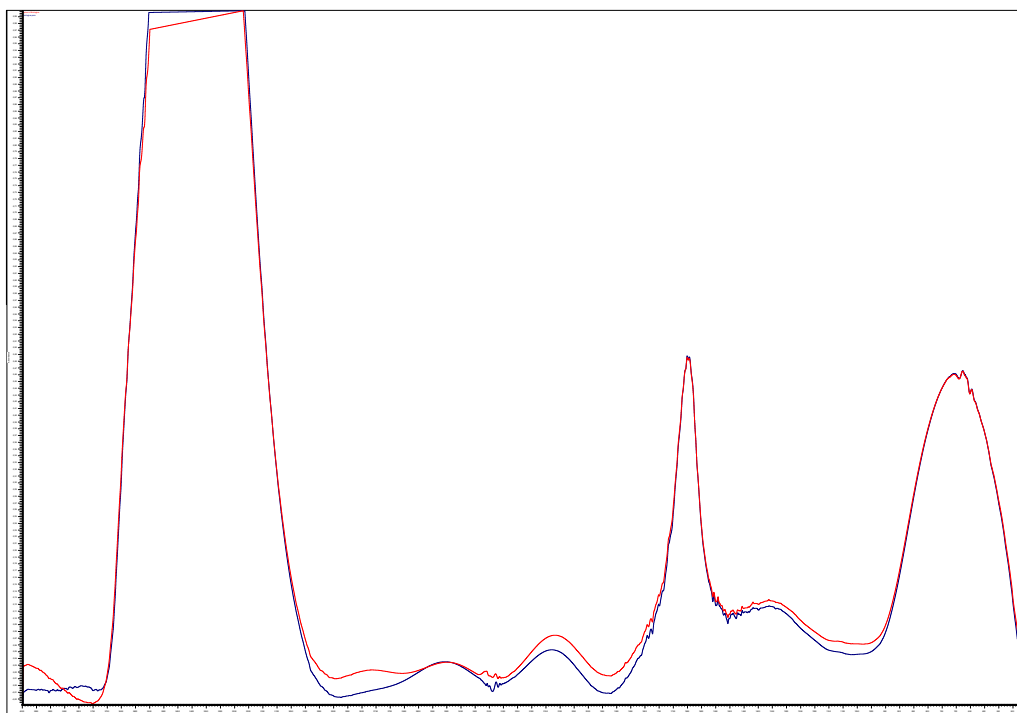


Figure 29 - Comparison between FT-IR spectra of saline solution (blue) and reference standard in saline solution (red).

Comparison between the extraction media

A qualitative comparison of samples extracted in ethanol and in physiological saline solution showed no differences in the presence of bisphenol-derived spectral features. In both cases, the spectra were dominated by solvent-related contributions and residual baseline noise after background subtraction. Differences observed between the two extraction media were limited to variations in band intensity, especially in the O–H stretching region, which appeared more pronounced in saline extracts due to their higher water content and hydrogen-bonding capacity. These variations result from differences in solvent polarity and residual moisture retention and were not associated with the appearance of new absorption bands or diagnostic features. Overall, FT-IR analysis indicates that the type of extraction solvent does not qualitatively influence the detectability of bisphenols, supporting the conclusion that no bisphenol-related infrared signatures are present in the tested samples under the experimental conditions used. (Figures 30 and 31)

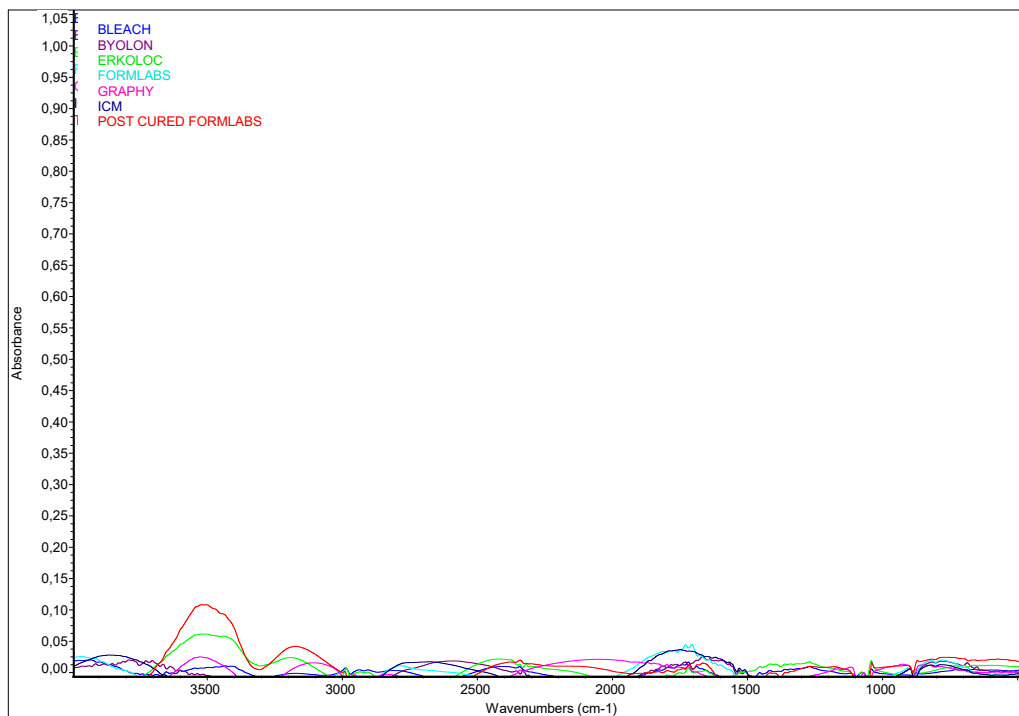


Figure 30 - Samples in EtOH

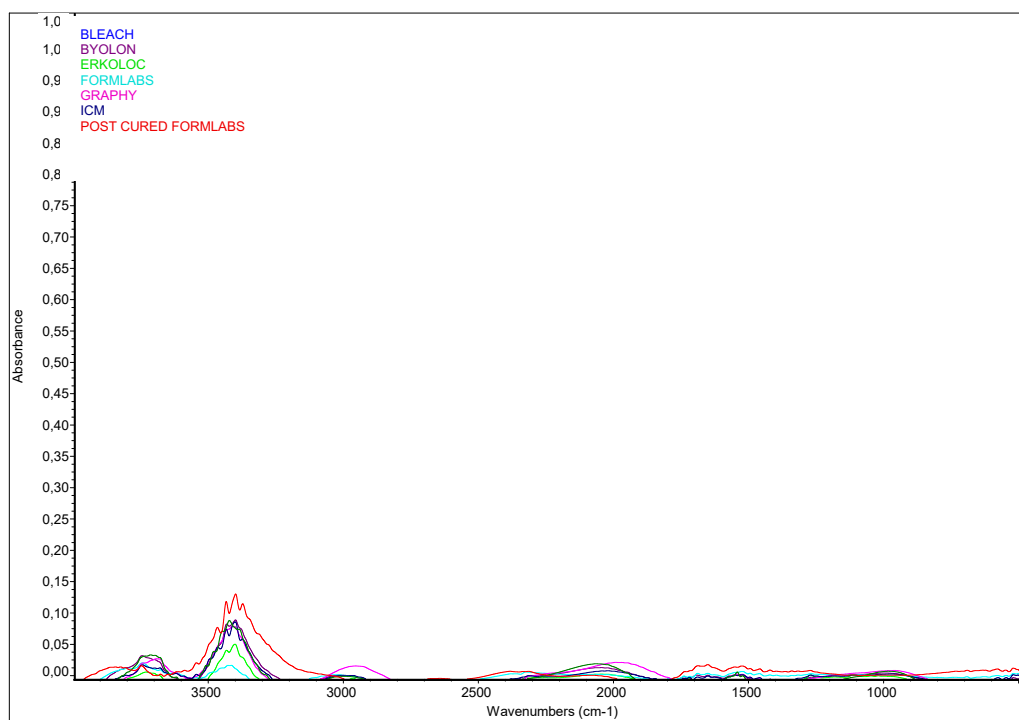


Figure 31- Samples in NaCl

GC–MS Results

GC–MS analysis was performed to investigate the potential release of bisphenols and related compounds from orthodontic polymeric materials after extended incubation in various extraction media. All samples were analysed after derivatisation of phenolic hydroxyl groups, which allowed for accurate chromatographic separation and mass spectrometric detection.

Verification of derivatisation and method performance

The effectiveness of the derivatisation and extraction procedure was initially assessed using reference standards and BPA-spiked samples. Analysis of derivatised standards produced well-defined chromatographic peaks with symmetrical profiles and consistent retention times. The corresponding EI mass spectra displayed characteristic fragmentation patterns matching reference data. Chromatographic analysis of unspiked physiological saline and ethanol extracts did not detect peaks attributable to bisphenols above the method's detection limit. (Figure 32)

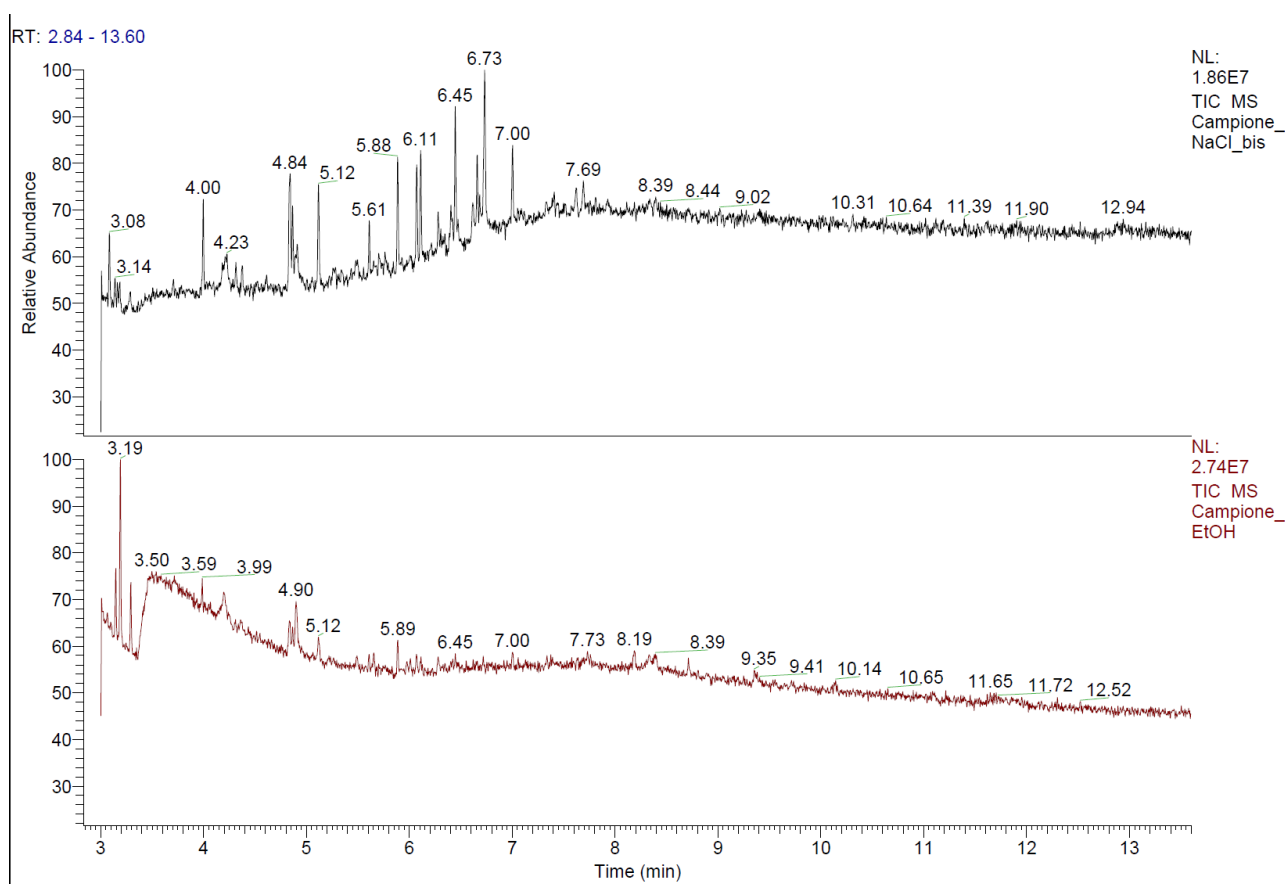


Figure 32 - GC-MS chromatograms of physiological saline (top) and ethanol (bottom) extracts after derivatisation, showing no detectable peaks attributable to bisphenols.

In contrast, BPA-spiked samples consistently showed a single, well-separated chromatographic peak corresponding to derivatised BPA (Figure 33), with retention time and mass spectral features fully aligning with those of the reference standard. These observations confirmed the suitability of the analytical workflow for BPA detection under the adopted conditions.

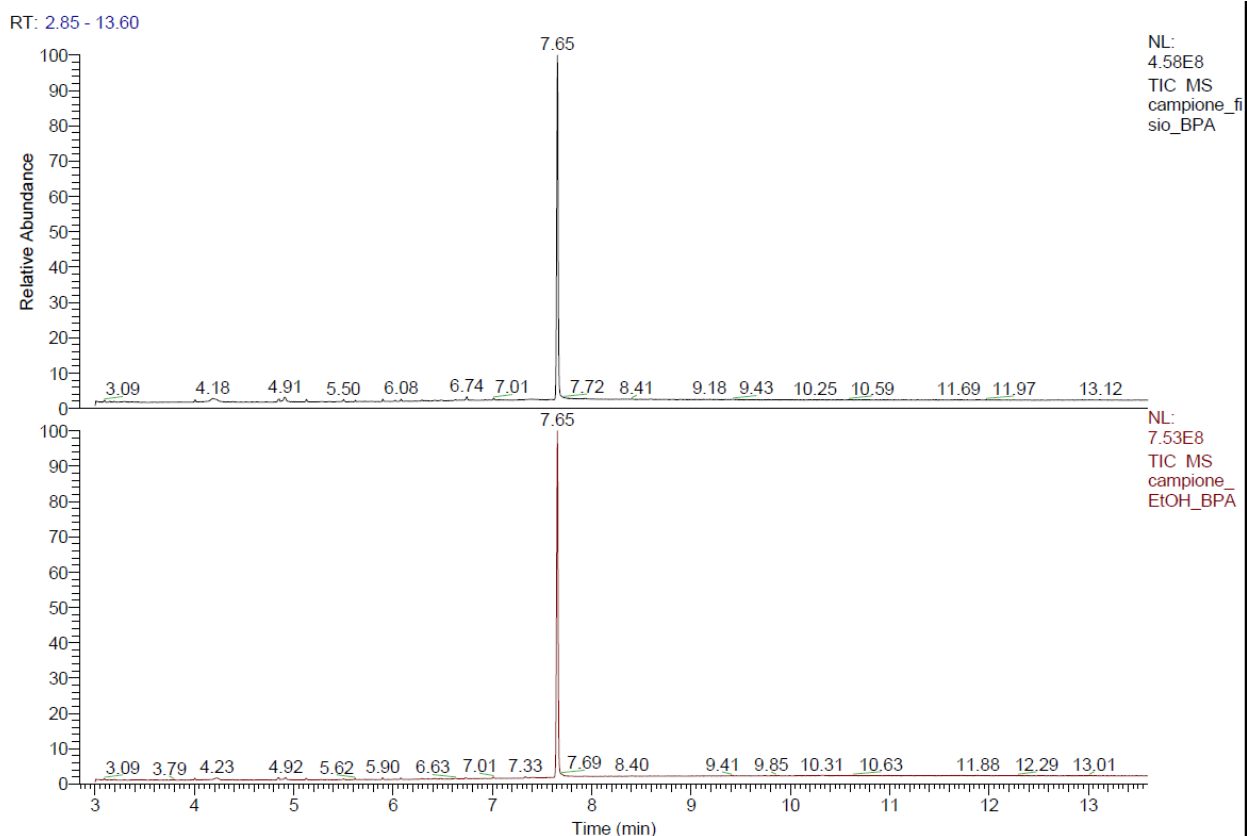


Figure 33 - GC-MS chromatograms of physiological saline and ethanol extracts spiked with bisphenol A, confirming effective derivatisation and method sensitivity.

Analysis of ethanol extracts

In one experimental series, GC-MS analysis of ethanol extracts revealed a very faint chromatographic signal attributable to derivatised BPA. Identification was based on matching retention time and mass spectral fragmentation pattern with those obtained from reference standards and spiked samples. Repeated injections of the same extract yielded similar results, indicating acceptable instrument stability. A tentative semi-quantitative estimate, based on comparing peak areas with those observed in spiked samples, indicated a bisphenol quantity of approximately 0.005 μg per polymer disc. This estimate should be interpreted with caution, as it was obtained without a calibration curve and from a limited number of analytical replicates. Notably, the signal was not consistently reproducible in subsequent independent experiments conducted under identical incubation and analytical conditions.

Due to its very low intensity and limited reproducibility, the occasional detection of BPA in ethanol extracts was cautiously interpreted as more likely caused by experimental contamination or analytical artefacts rather than a consistent release from the tested polymeric materials (Figure 34)..

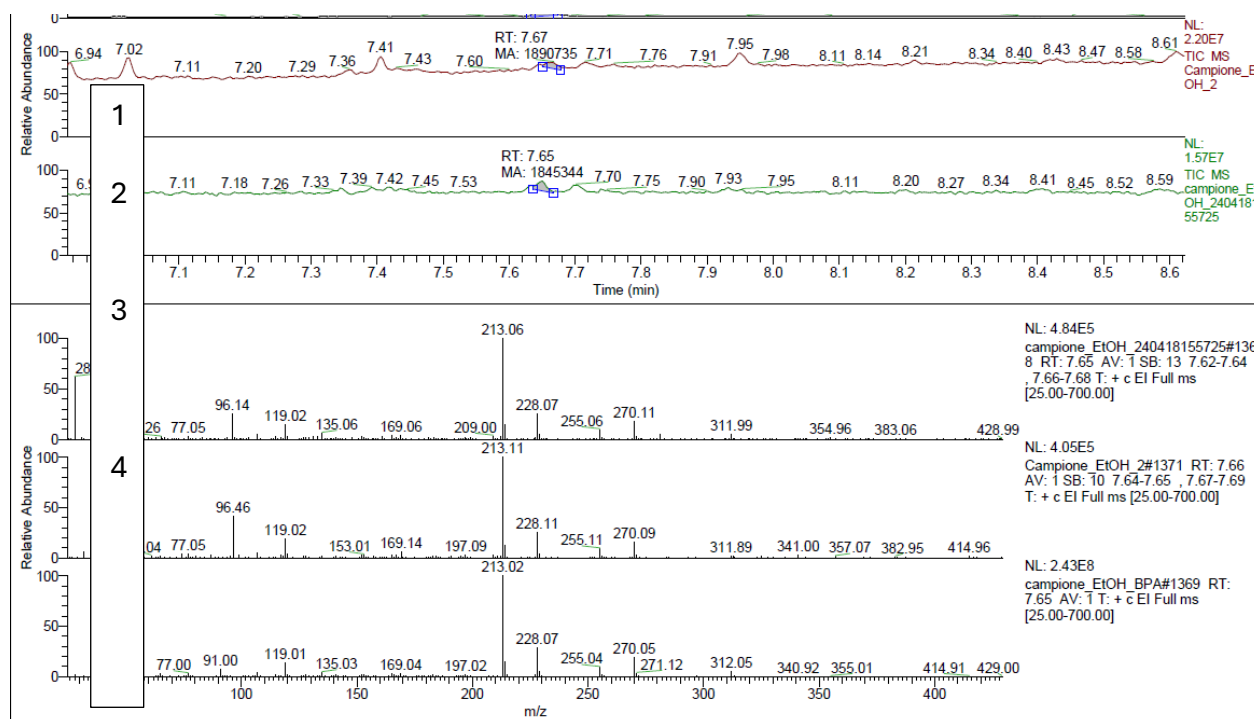


Figure 34 - Gas chromatogram of (1) the EtOH extract spiked with BPA; (2) the unspiked EtOH extract, first aliquot analysis, and (3) the unspiked EtOH extract second aliquot analysis. (4) Mass spectra perfectly matching the spectra of di-acetylated BPA in 1,2 and

Identification of bisphenol A was achieved through the combined analysis of chromatographic retention behaviour and electron ionisation mass mass-spectral fragmentation. Under the chromatographic conditions used, derivatised BPA consistently eluted at approximately 7.6–7.7 minutes. The EI mass spectrum displayed the characteristic fragment ions expected for diacetylated BPA, fully aligning with reference standards and library spectra. No additional chromatographic signals with matching retention behaviour or diagnostic fragmentation patterns attributable to other bisphenol analogues were detected in either ethanol or physiological saline extracts. Based on this, the presence of bisphenol-related compounds other than BPA could be ruled out within the method's detection limits.

Results of the MCF-7 Assay

MCF-7 was initially intended as a third-level screening; after obtaining negative analytical results, it was used as a targeted confirmatory assay on representative samples. Based on the FT-IR and GC–MS analyses, which did not detect bisphenols or other estrogenic compounds in the extracts, a comprehensive biological screening of all investigated polymeric materials was not conducted. Instead, five polymeric materials were randomly selected, extracted, and then evaluated using the MCF-7 cell proliferation assay. This approach aimed to assess the overall cellular response to representative materials and to verify the potential occurrence of cytotoxic or oestrogen-like effects at the cellular level in the absence of chemically detectable oestrogenic substances.

Exposure of MCF-7 cells to 17β -estradiol caused a clear and reproducible increase in cell proliferation compared with the negative control. The proliferative response was observed across the tested concentration range, confirming the oestrogen responsiveness and the functional integrity of the experimental model (Figure 35). These results confirm the suitability of the MCF-7 proliferation assay for detecting oestrogen receptor-mediated biological effects under the conditions used.

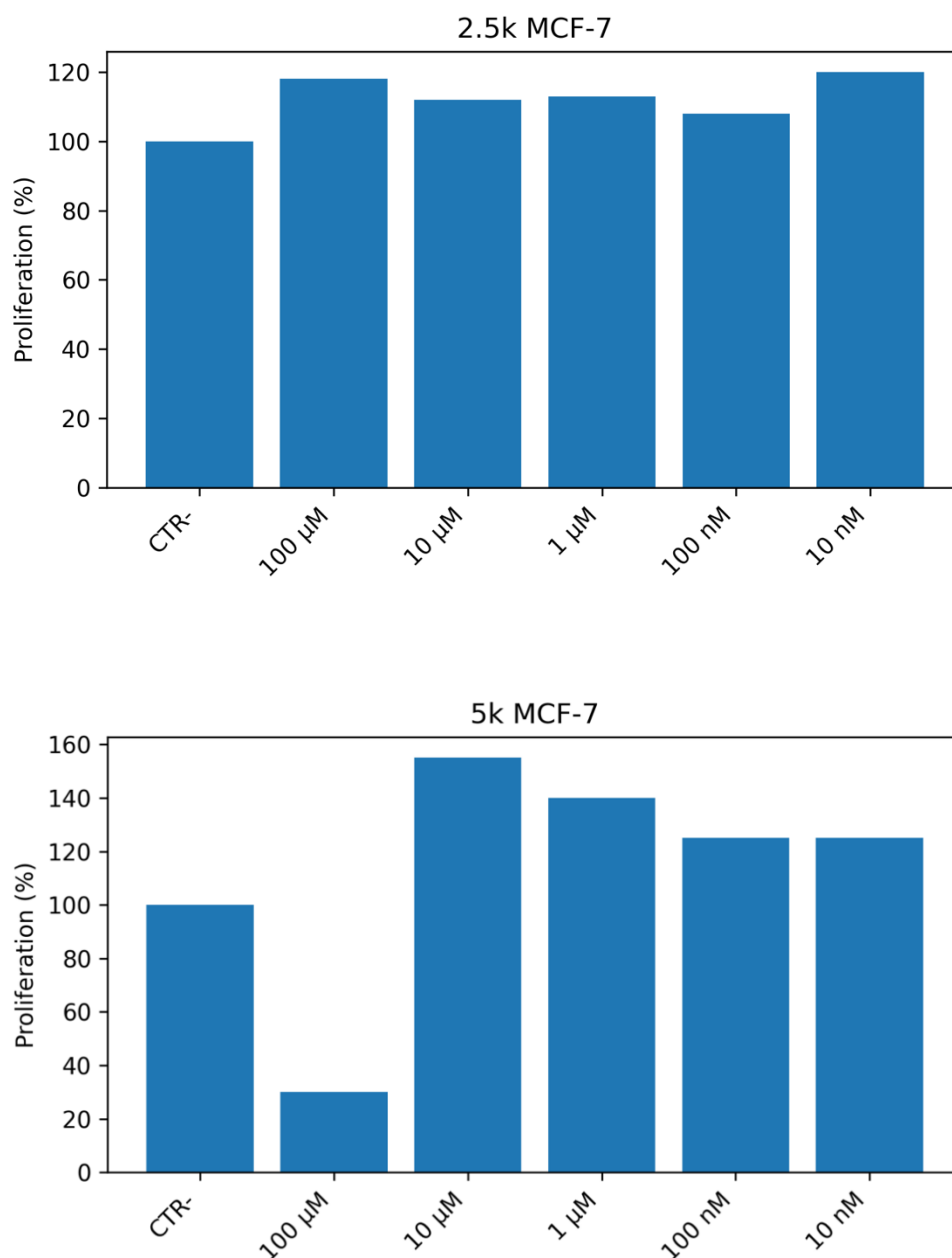


Figure 35 - Model Validation of the proliferative response of MCF-7 cells to increasing concentrations of 17β -estradiol

Effect of NaCl solution on cell proliferation

In the initial experimental series, exposing MCF-7 cells to NaCl-extraction solutions resulted in a significant decrease in cell proliferation compared to the negative control. This effect was consistently seen with all tested polymeric materials and showed no material-specific differences. Control experiments using only physiological saline solution without any polymer contact produced a similar

decrease in cell proliferation. However, when the saline solution was dried and then reconstituted before exposing the cells, proliferation levels nearly matched those of the control group (Figures 36 and 37). These results suggest that the reduction in proliferation under saline exposure is due to the saline matrix, not to polymer-derived substances.

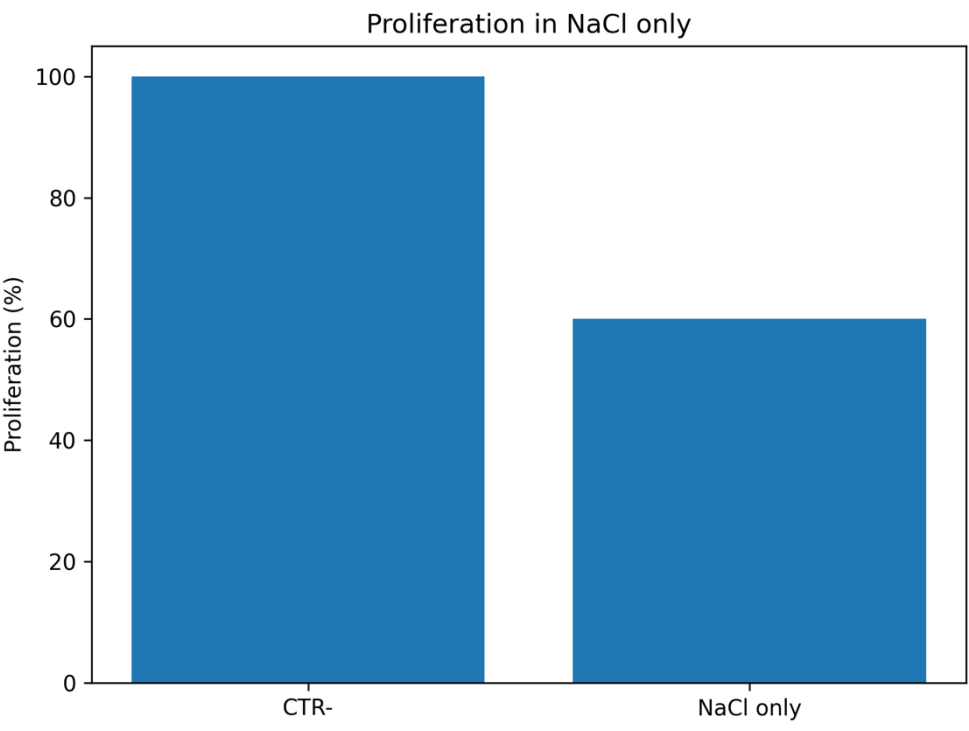


Figure 36 - Effect of saline solution exposure on MCF-7 cell proliferation in the absence of polymer extracts.

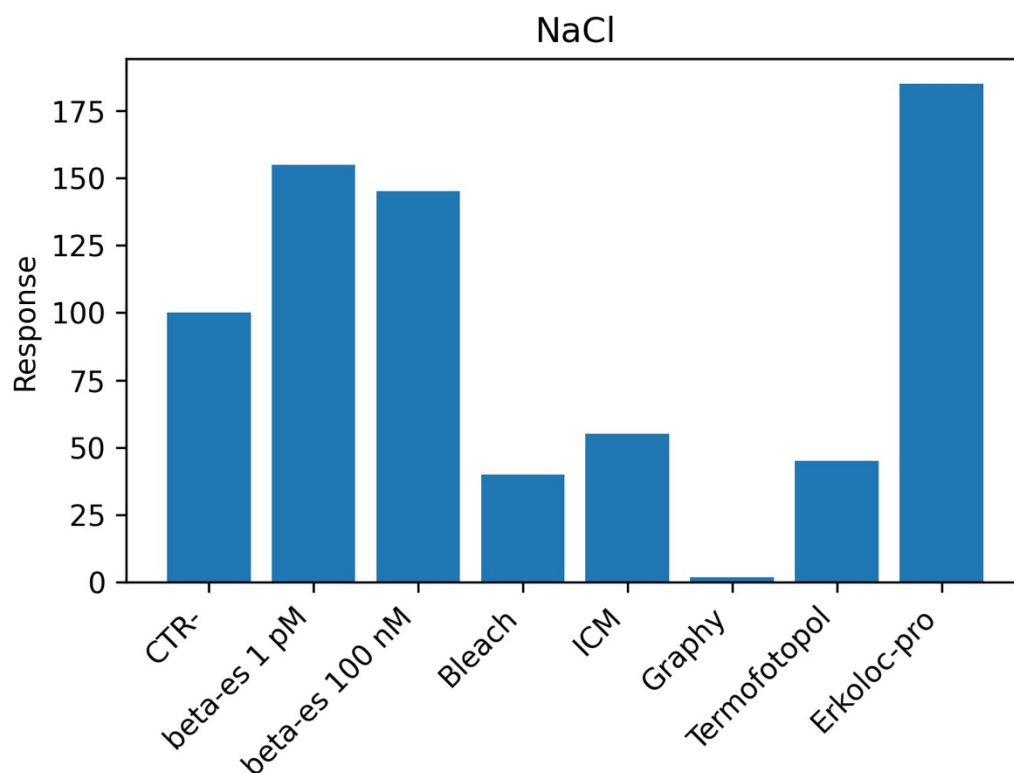


Figure 37 - Effect of saline solution exposure on MCF-7 cell proliferation in the presence of polymer extracts.

Effect of ethanol on cell proliferation

Exposure of MCF-7 cells to ethanol resulted in a moderate increase in cell proliferation compared with the negative control. This effect was seen both under control conditions and in samples from polymer extraction in ethanol. The proliferative response was limited and did not show a clear concentration-dependent trend (Figures 38 and 39).

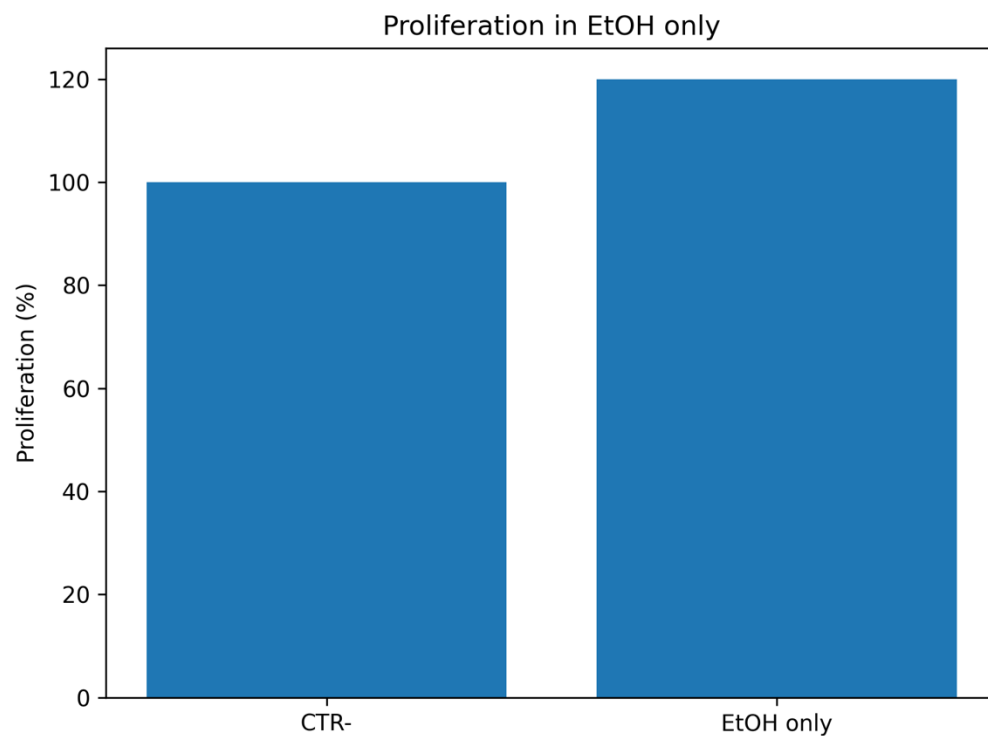


Figure 38 - Effect of ethanol exposure on MCF-7 cell proliferation in the absence of polymer extracts.

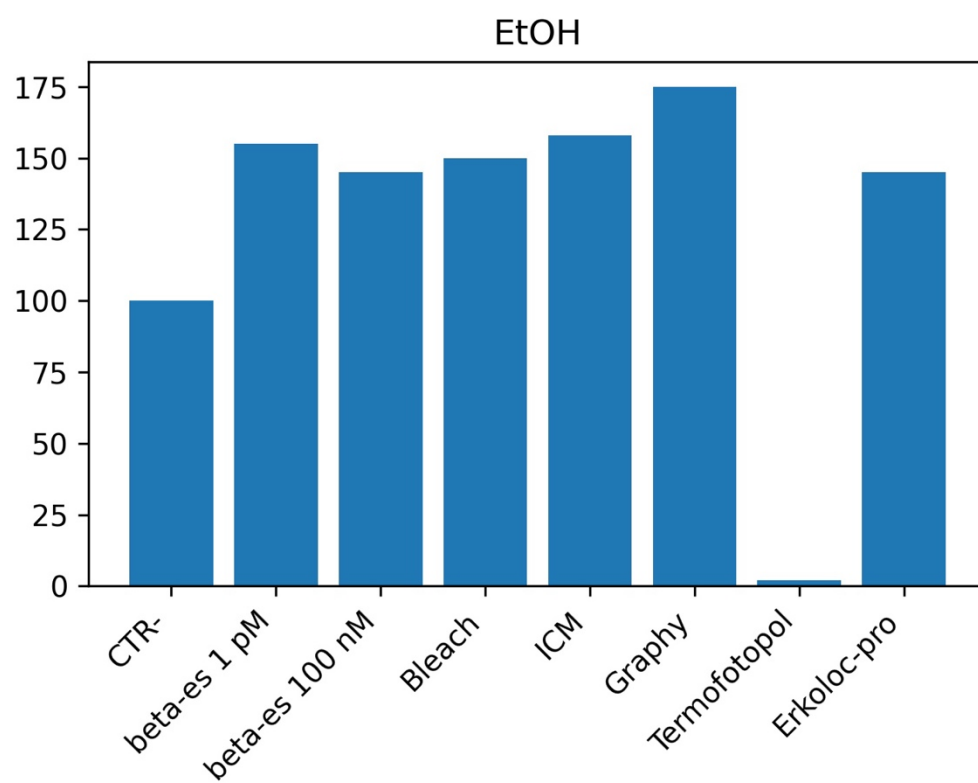


Figure 39 - Effect of ethanol exposure on MCF-7 cell proliferation in the presence of polymer extracts.

No qualitative differences were observed between ethanol controls and ethanol-based polymer extracts, indicating that the proliferative effect was not specifically linked to the polymeric materials.

When evaluating the responses caused by derived extracts in relation to the corresponding solvent controls and positive controls, no evidence of estrogenic activity attributable to the investigated polymeric materials was observed. None of the extracts induced a proliferative response comparable to that elicited by 17 β -estradiol, nor did they exhibit concentration-dependent behaviour consistent with oestrogen receptor-mediated stimulation.

Across all experimental conditions, the observed cellular responses could be attributed to the inherent effects of the extraction media, specifically the growth-inhibitory effect associated with physiological saline solution and the moderate proliferative effect linked to ethanol exposure.

DISCUSSION

This research project investigated the biocompatibility of materials used in orthodontic clear aligners through an integrated, step-by-step experimental approach. The biological, clinical, and patient satisfaction aspects of CAT have been investigated. However, the biological effects of long-term intraoral exposure to polymeric devices remain insufficiently understood (Iliadi et al., 2020; Tamburrino et al., 2020). Therefore, this study aims to evaluate the safety profile of orthodontic materials through a combination of structural, chemical, and biological analyses. Clear aligners are worn for over 20 hours daily and are periodically replaced. CAT can last from several months to years (Meade et al., 2022; Meade et al., 2024). Consequently, there is prolonged and repeated contact with oral tissues in an environment influenced by temperature, pH, mechanical stress, and enzymes. According to the literature, these conditions may accelerate material ageing and cause the release of low-molecular-weight substances (Brandão et al., 2024; Elkholy et al., 2019).

This issue is especially concerning in younger patients, who are biologically more sensitive and often require long-term aligner therapy (Iliadi et al., 2020). It is also important to emphasise that in vivo exposure to substances released from orthodontic materials occurs alongside ongoing dietary intake. Bisphenols and related compounds are common environmental contaminants, with diet being the primary source of exposure for most people, often exceeding contributions from medical or dental devices (Stahlhut et al., 2009; Venisse et al., 2014). Assessing systemic exposure from orthodontic appliances in vivo is particularly challenging and represents a major limitation of biomonitoring studies. Therefore, this study highlights the need to consider material safety as part of the overall evaluation of CAT, rather than focusing solely on biomechanical efficacy. The main hypothesis suggests that polymeric materials used in aligner production may undergo structural changes over time.

This could enhance the release of bioactive substances, such as bisphenols and related compounds. Furthermore, it is hypothesised that these substances might exert estrogen-like biological effects, even if not detectable by standard analytical techniques (Bittner et al., 2014; Rochester & Bolden, 2015). To investigate this, a tiered analysis was employed to study how bisphenols and related compounds are released. Samples were aged in solution before analysis. Two solvents with different polarities were used for extraction, enabling a broader range of molecules to be extracted (Caballero-Casero et al., 2016; Yang et al., 2011). Polar solvents, such as saline, extract water-soluble (hydrophilic) compounds, while less polar solvents, like ethanol, extract lipid-soluble (lipophilic) substances that are commonly found in plastics. In this study, a NaCl solution simulated clinical conditions, while ethanol offered greater extraction capacity. Using both solvents allowed us to differentiate between normal physiological release and maximum possible extraction, thereby increasing the detection of all releasable substances. Using multiple solvents improves the detection of estrogen-like molecules, as no single solvent extracts all compounds (Yang et al., 2011).

Despite employing chemically differentiated extraction conditions, the ageing protocol in this study was a highly simplified model of intraoral conditions. Chemical ageing was simulated using solvents of varying polarity at controlled temperatures. However, mechanical wear was not directly incorporated. Clinically, aligners are in friction with teeth and occlusal forces. They undergo cycles of insertion and removal, as well as exposure to parafunctional habits. These factors may lead to surface degradation and material fatigue (Koenig et al., 2022; Wulff et al., 2022), but were not examined in the present study.

The initial analysis used FT-IR spectroscopy to examine chemical changes after exposure to different solvents and ageing. No bisphenols or similar molecules were found, showing that FT-IR cannot detect small amounts of additives in complex plastics (Dalaie et al., 2021; Khijmatgar et al., 2022). These results should be viewed alongside FT-IR's limitations: it can analyse complex mixtures but

does not efficiently identify individual molecules. Notably, clear yet consistent patterns appeared between thermoplastics and printed resins, likely reflecting differences in polymer makeup and manufacturing methods (Kolenc et al., 2025). Overall, the results suggest that material type and production affect both ageing and substance release.

To overcome FT-IR's limitations, subsequent analysis was conducted using GC-MS, a more sensitive second-tier analytical technique. GC-MS generally detected low or undetectable levels of bisphenols under most conditions, particularly in NaCl extracts. Ethanol showed greater extraction efficiency. These findings align with previous studies emphasising the impact of solvent polarity on extractable profiles (Kotyk & Wiltshire, 2014; Yang et al., 2011). However, it is important to recognise that GC-MS is a targeted analytical method that focuses on predefined analytes. It does not capture the full complexity of polymer degradation products. This is a significant limitation when assessing materials that may release diverse compounds, including oligomers, additives, and degradation fragments not included in analytical standards (Dantagnan et al., 2025; Pratsinis et al., 2022).

Considering these experimental conditions, polymer decay can produce heterogeneous mixtures. Their biological activity is determined by cumulative effects rather than individual compound concentrations (Rosenfeld, 2017). Integrating physical and chemical analyses with a biological assay was a key methodological step. Using an estrogen-responsive MCF-7 proliferation assay allowed assessment of the extracts' estrogenic potential. This approach is supported by toxicological evidence. Studies show that biological activity may occur even when individual compounds are below analytical detection limits (Caballero-Casero et al., 2016; Rosenfeld, 2017). The MCF-7 proliferation assay found no estrogenic activity attributable to the polymer extracts in this study. Variations in cell proliferation were entirely due to solvent-related effects. There was no specific biological contribution from the polymeric materials. These results align with existing literature, emphasising the importance of interpreting *in vitro* estrogenicity assays with solvent controls and considering

model-specific limitations (Heindel et al., 2020). It is also important to note that the MCF-7 cell line is a simplified biological model. The absence of estrogenic activity in vitro does not rule out subtle or long-term effects in clinical settings (Ferreira et al., 2025; Rosenfeld, 2017). In this study, no estrogenic activity attributable to polymer extracts was observed. Variations in cell proliferation were solely due to solvent-related effects, with no specific biological contribution from the polymeric materials. These findings are consistent with the literature, which highlights the need to analyse in vitro estrogenicity assays considering solvent controls and model-specific limitations (Heindel et al., 2020).

Despite these limitations, the primary strength of this study is the integration of structural, chemical, and biological data. Combining FT-IR, GC-MS, and MCF-7 assays provides a more comprehensive and reliable safety assessment than relying on a single analytical technique (Iliadi et al., 2020). Clinically, although this in vitro study did not indicate acute risks from aligner use, it emphasises the importance of considering chronic low-dose exposure in relation to treatment duration and patient age in clinical decision-making. This is particularly relevant during long-term orthodontic therapy (Meade et al., 2023). Recent in vivo and biomonitoring studies suggest that orthodontic appliances may contribute minimally to systemic exposure to polymer-derived substances. However, differentiating this exposure from predominant dietary sources remains methodologically challenging (Stahlhut et al., 2009; Venisse et al., 2014). Collectively, these statements underscore the value of controlled in vitro models and the necessity for cautious interpretation and further in vivo research.

Limitations of the study

Nevertheless, despite its innovative design, the study has limitations concerning in vitro modelling, material choice, and simplified ageing conditions. Specifically, the ageing protocol focused on chemical exposure and did not include mechanical wear or abrasion, which are increasingly recognised as important factors in polymer degradation and microplastic formation in the oral

environment (Torkomian et al., 2025; Wulff et al., 2022). Recent evidence shows that microplastics produced by mechanical wear may influence both local and systemic toxicity, acting as physical particles and as carriers for adsorbed chemical substances (Koenig et al., 2022; Zecca et al., 2025). These aspects were outside the scope of this study but remain a critical topic for future research.

Although using an aqueous NaCl solution helps mimic clinically relevant conditions, employing artificial saliva or simpler aqueous media presents an inherent limitation of in vitro oral exposure models. Artificial saliva formulations cannot fully replicate the biological and physicochemical complexity of human saliva, which contains proteins, mucins, enzymes, and antimicrobial components that influence lubrication, buffering capacity, pellicle formation, and material–tissue interactions (Łysik et al., 2019). Consequently, enzymatic activity and rheological properties relevant to polymer degradation and substance release may not be accurately reproduced. Some studies have promoted the use of artificial saliva for evaluating materials for intraoral use without considering additional limitations. Artificial saliva formulations cannot entirely replicate the biological and physicochemical complexity of human saliva, which includes proteins, mucins, enzymes, and antimicrobial components affecting lubrication, buffering capacity, pellicle formation, and material–tissue interactions (Łysik et al., 2019). As a result, enzymatic activity and rheological properties related to polymer degradation and substance release may not be adequately simulated. Furthermore, human saliva exhibits significant inter- and intra-individual variability, influenced by physiological and environmental factors, leading to differences in composition and enzymatic activity that standardised artificial formulations cannot capture (Wong & Sissions, 2001). Additionally, evidence from oral biofilm studies suggests that chemically defined saliva analogues do not fully substitute biologically representative systems, advising caution when extrapolating in vitro results to the dynamic intraoral environment (Wong & Sissions, 2001). Despite these limitations, the approach's conceptual robustness remained intact; indeed, it provided a foundation for future research directions. These should encompass dynamic ageing models that integrate mechanical stress, artificial saliva

systems, microplastic analysis, and in vivo investigations, as increasingly recommended in recent literature (Ferreira et al., 2025)

Future Perspectives

Future research on aligner biocompatibility should adopt experimental designs that reflect the complexity of real clinical conditions. While in vitro models remain essential for isolating material-specific effects and controlling experimental variables, they cannot fully replicate the dynamic oral environment. Therefore, in vivo studies are a vital next step to validate and contextualise the findings obtained under controlled laboratory conditions. Specifically, future studies should develop combined ageing protocols that incorporate both chemical and mechanical wear, mirroring the simultaneous exposure of aligners in clinical use to saliva, temperature fluctuations, pH variations, occlusal forces, and repeated insertion and removal cycles. The interaction between chemical degradation and mechanical abrasion can substantially influence material stability, surface integrity, and the release profile of both soluble compounds and particulate debris, including micro- and nano plastics. Developing dynamic in vitro models that include artificial saliva, cyclic mechanical loading, and long-term exposure protocols may serve as an important intermediate step. These models would enable more realistic simulation of intraoral conditions while maintaining experimental control, thereby improving the predictive value of laboratory studies and guiding subsequent clinical research. Finally, in vivo biomonitoring studies in orthodontic patients that assess salivary and urinary biomarkers over the long term are necessary to evaluate the true contribution of aligner materials to systemic exposure, while accounting for confounding factors such as diet and environmental sources. Integrating chemical, biological, and clinical data will be essential for creating a comprehensive, clinically relevant risk assessment framework for both current and emerging orthodontic materials.

CONCLUSIONS

This research project involved a comprehensive, multi-level assessment of the chemical and biological safety of materials used in orthodontic clear aligners. Thermoplastics and moulded resins were analysed to assess their behaviour under simulated ageing conditions in polar and non-polar solvents. The methodological approach, which combined spectroscopic techniques, targeted chemical analyses, and functional biological assays, aimed to address the limitations of previous studies that focused solely on identifying single chemical markers. The results indicate that the polymer composition and ageing conditions have a slight effect on the stability of the materials and the release of bisphenols and related compounds.

FT-IR and GC–MS analysis revealed limited or variable detectability of individual bisphenols, especially in alcoholic solutions. This was confirmed by biological assays in MCF-7 cells, which showed that the complex extracts do not exhibit estrogen-like biological activity. These findings emphasise the importance of combining chemical and biological assessments to gain a more precise understanding of the risks associated with long-term clinical use of aligners. Overall, this research advances knowledge of the safety profile of polymeric orthodontic devices and provides a solid scientific basis for evidence-based clinical decisions and the future development of safer, more effective materials.

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