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ORIGINAL ARTICLE

A comparative in vitro study on monomer release from bisphenol A-free and conventional temporary crown and bridge materials

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Abstract

This study aimed to investigate the release of common monomers from two conventional and two bisphenol A (BPA)-free temporary crown and bridge materials. Cylindrical samples of all materials were prepared ($N = 90$; five samples for each material and cycle of analysis). All samples were immersed in high-performance liquid chromatography (HPLC)-grade water and incubated for 1 h, 12 h, 24 h, and 7 days in an incubation shaker at 37˚C and 112 rpm. Extraction was performed in accordance with ISO 10993-12. Eluted monomers were detected and quantified by HPLC coupled with ultraviolet–visible spectroscopy and mass spectrometry (HPLC-UV/Vis-MS). Analysis of BPA was performed by HPLC coupled with ultraviolet–visible spectroscopy (HPLC-UV/Vis) and positive results were verified by HPLC-tandem mass spectrometry (HPLC-MS/MS). Neither bisphenol A-glycidyl methacrylate (Bis-GMA) nor BPA was quantifiable in any of the crown and bridge samples investigated in the present study. However, all samples contained triethylene glycol dimethacrylate (TEGDMA) and/or urethane dimethacrylate (UDMA) after 24 h of incubation. Statistical analysis showed that significantly more UDMA was released from the BPA-free materials than from the conventional materials. All concentrations of UDMA measured were below the effective cytotoxic concentrations previously reported. However, for a few materials, especially BPA-free temporary crown and bridge materials, the levels of UDMA were above previously reported potentially harmful concentrations for local cells. As BPA-free materials were introduced as being more biocompatible than materials containing BPA, substitution of Bis-GMA with UDMA should be further investigated.

KEYWORDS
biocompatibility Testing, bisphenol A-glycidyl methacrylate, dental materials, dental restoration, materials testing, polymers

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INTRODUCTION

Temporary restorations play an integral part in most prosthetic treatment courses as they are used from tooth preparation until placement of the final restoration [1]. During this time, temporary crowns and bridges serve to maintain the esthetics and functionality of the masticatory system and to protect teeth from thermal, mechanical, and microbial noxae [2,3]. In prosthetic dentistry, temporary restorations are fabricated with resin-based crown and bridge (C&B) materials, which can be subdivided into methacrylate resins (liquid/powder, hand-mixed) and composite resin-based materials [4,5]. As a result of their mechanical and esthetic properties, composites are currently being used as state-of-the-art materials [5,6]. The organic polymer matrix of C&B composite materials is very similar to that of composites used for dental fillings [7]. The monomers predominantly used are bisphenol A-glycidyl methacrylate (Bis-GMA), urethane dimethacrylate (UDMA), and an additional co-monomer, which is usually triethylene glycol dimethacrylate (TEGDMA) [8–10].

In recent years, the release of residual monomers, and especially bisphenol A (BPA), from dental composites has been a cause for public concern [11]. However, pure BPA is not being used as a monomer in dentistry, and thus only small amounts are leachable as a result of possible contamination from the use of BPA derivatives [12,13]. Composites for restoring dental cavities are similar to C&B composites in terms of composition but differ with regard to the initiator system used, as the latter is self-curing or dual-curing and not light-curing [14].

In contrast to light-cured materials, C&B materials show the disadvantages of self-curing composites, such as a lower degree of conversion and air porosities [15–17]. Prior studies on core build-up materials have shown that insufficiently converted composites lead to sustained monomer elution [18–20]. As a result, some manufacturers have substituted BPA derivatives with UDMA and have introduced BPA-free composites in order to avoid the release of BPA and its derivatives [21–23]. As monomer elution depends on the extraction ratio (the ratio of surface area to solvent volume) [24–26], the larger surface area of temporary crowns and bridges compared with that of fillings and the use of self- or dual-curing materials could increase biocompatibility concerns. Therefore, this study aimed to examine the monomers released from C&B materials and to compare the concentrations of monomers released from conventional and BPA-free materials. The in vitro set up applied was intended to simulate clinical practice as closely as possible. In accordance with the clinical workflow, all samples were immersed in water immediately after preparation and incubated at 37°C, with mild agitation, to simulate the oral environment. Almost identical concentrations of monomers were released from samples incubated in water compared with those incubated in artificial saliva [27,28], whereas monomer release was increased from samples incubated in strongermedia, such as ethanol/water mixtures [26,27,29], which is attributed to the softening of Bis-GMA-based resins and the formation of soluble units [30–32]. Instead, stronger media are better suited for assessing potential long-term health risks [33]. Although the release of BPA and its derivatives is the focus of current research and these substances are considered a potential health risk [34–36], it is known that small quantities, at most, of Bis-GMA and BPA are released in aqueous media, such as water, artificial saliva, or collected saliva [26–28]. As the investigated materials are present in the oral cavity only for a short period of time and temporary restorations are luted directly after fabrication [37], water was used as the extraction medium in the present study and all samples were immersed in water immediately after preparation. The tested null hypotheses were: (a) monomer/BPA elution is material dependent; and (b) BPA and Bis-GMA are either not released or released only in very small amounts.

MATERIAL AND METHODS

Study design

To test the stated hypotheses, samples of four different C&B materials were prepared according to the manufacturers' specifications (discussed in the next section), then immersed immediately in high-performance liquid chromatography (HPLC)-grade water (Sigma Aldrich). The incubation time-periods were 1 h, 12 h, 24 h, and 7 days. Prior to calibration with the respective reference substances (Table 1), all eluates were analyzed for Bis-GMA, TEGDMA, and UDMA by high-performance liquid chromatography coupled with ultraviolet-visible spectroscopy and mass spectrometry (HPLC-UV/Vis-MS) and for BPA by high-performance liquid chromatography coupled with ultraviolet-visible spectroscopy (HPLC-UV/Vis) with confirmation by HPLC-tandem mass spectrometry (HPLC-MS/MS). Because of the high costs of HPLC-UV/Vis-MS measurements, the period of maximum elution corresponding to 24h of incubation according to the current literature was analyzed first. The absence of a monomer after 24 h of incubation was considered as sufficient evidence that the substance was not released from the material being examined. However, if a monomer was detected, all incubation periods were investigated to evaluate the respective elution patterns. Subsequently, the results were statistically evaluated in order to reveal potential material-dependent differences.

Sample preparation

According to the manufacturer's specifications, standardized cylindrical samples were prepared of two conventional temporary C&B materials, Protemp 4 (3 M ESPE) and Luxatemp Automix Plus (DMG), and of two BPA-free materials, ExperTemp (Ultradent), and Visalys Temp (Kettenbach).

TABLE 1 Chemicals used for derivatization and high-performance liquid chromatography (HPLC) analysis

Name	Abbreviation	Manufacturer	Molecular mass (g mol^{-1})	CAS-Nr.	Purity
Urethane dimethacrylate	UDMA	Sigma Aldrich	470.56	72869-86-4	> 97%
Triethylene glycol dimethacrylate	TEGDMA	Sigma Aldrich	286.32	109-16-0	99%
Bisphenol A	BPA	Sigma Aldrich	228.29	80-05-7	≥ 99%
Bisphenol A-glycidyl methacrylate	Bis-GMA	Sigma Aldrich	512.59	1565-94-2	Not specified
Diethyl phthalate	DEP	Sigma Aldrich	222.24	84-66-2	99.5%
Pyridine-3-sulfonyl chloride	PSC	Sigma Aldrich	177.61	16133-25-8	≥ 98.0%
Bisphenol A-d16	d16BPA	Sigma Aldrich	244.38	96210-87-6	98 atom %

TABLE 2 Composition of the crown and bridge materials tested

Material	Main monomers*	Contains BPA*	Manufacturer
ExperTemp	Aliphatic dimethacrylate, poly(alkylene glycol) diacrylate, hydroquinone monomethyl ether	BPA free	Ultradent
Luxatemp Automix Plus	Dimethacrylate	–	DMG
Protemp 4	Dimethacrylate, reaction products of 1,6-diisocyantohexane with 2-[(2-methacryloyl)ethyl]6-hydroxyhexanoate and 2-hydroxyethyl methacrylate	–	3 M ESPE
Visalys Temp	Aliphatic dimethacrylate, poly(alkyleneglycol)diacrylate, hydroquinone monomethyl ether	BPA free	Kettenbach

*According to manufacturers' information.

Information about the composition of the C&B materials is given in Table 2. Chemical polymerization was initiated by placing all samples in polytetrafluorethylene (PTFE) molds (10 mm diameter; 10 mm height) using an automix dual cartridge. Excess material was removed using a glass slide and the samples were left undisturbed for the recommended setting duration (4 min for ExperTemp,7 min for Luxatemp Automix Plus, 5 min for Protemp 4, and 4 min for Visalys Temp). This resulted in samples ($n = 5$ for each combination of material and incubation period) with a surface area of 4.712 cm^2. Following the manufacturer's instructions, all samples were washed with ethanol to remove the oxygen inhibited layer. For standardization, the samples were fully immersed in ethanol and agitated for 20 s. To remove excess ethanol, the samples were cleaned with HPLC-grade water.

Incubation

Ultraviolet light-protected borosilicate sample containers with PTFE-coated closures were used for sample incubation. Each vial was cleaned using HPLC-grade methanol (Sigma Aldrich) and HPLC-grade water. Corresponding to the clinical workflow, all samples were immediately immersed in 1.74 mL of HPLC-grade water after preparation. The extraction ratio (surface area/solvent volume) was chosen following ISO 10993-12 and all samples were fully covered with HPLC-grade water. Incubation was performed in an incubator shaker (Excella E24; New Brunswick Scientific) for 1 h, 12 h, 24 h, and 7 days at 37°C and 112 rpm. Following each period of incubation, the samples were removed, and to prevent secondary chemical reactions the eluate was frozen at −18°C and kept in the dark.

HPLC-UV/Vis-MS analysis

After thawing at room temperature, aliquots of 0.5 mL of each eluate and 0.5 mL of the internal standard, diethyl phthalate (Sigma Aldrich) (10.0 μg mL^{-1}), were transferred into HPLC amber glass vials. For detection of BPA, an external calibration without diethyl phthalate was performed. The HPLC-UV/Vis-MS analysis was carried out on an Agilent 1200 SL (Agilent Technologies) with a Surveyor PDA Plus Detector

TABLE 3 Mass spectrometry settings (high-performance liquid chromatography coupled with ultraviolet–visible spectroscopy and mass spectrometry [HPLC-UV/Vis-MS])

Parameter	Setting
Ionization source	Electrospray ionization
Mass range	100–1000 *m/z*
Source voltage	4 kV
Capillary temperature	275°C
Capillary voltage	42 kV
Tube lens	125 V
Sheath gas flow	50 arb
Auxiliary gas flow	0 arb
Resolution	60 000

Abbreviations: arb, arbitrary unit; *m/z*, mass-to-charge ratio.

(Thermo Fisher Scientific) coupled to an LTQ Orbitrap XL with high-resolution MS capability (Thermo Fisher Scientific). Separation was performed in a Kinetex 100A column (Phenomenex) with 150 × 2.1 mm dimensions and a 5 μm particle size. The column was kept at 25°C, and the injection volume was 10 μL. Solvent A was water with 0.05% (v/v) formic acid, and solvent B was methanol with 0.05% (v/v) formic acid. Gradient elution was applied as follows: 0 min, 60% A; 0–15 min, 60%–0% A; 15–22 min, 0% A. The scan range of the photodiode array (PDA) was set to 200–600 nm with a scan rate of 1 nm for Bis-GMA, TEGDMA, and UDMA analysis. For BPA analysis, the scan range was adjusted to 270–280 nm. Mass spectrometric analysis for detection of Bis-GMA, TEGDMA, and UDMA was performed using electrospray ionization (ESI) (see Table 3 for a summary of all technical settings). All measurements were performed in duplicate.

As proposed by the American Chemical Society, the limit of detection (LOD) of all substances investigated was determined experimentally by measuring blanks and a dilution series (0.01 μg mL^{-1}, 0.05 μg mL^{-1}, and 0.5 μg mL) [38]. For all substances detected, the limit of quantification (LOQ) was determined by the lowest calibration standard on the calibration curve, as proposed by the European Medicines Agency [39]. The calibration curve of UDMA and TEGDMA consisted of five concentrations from 0.5 to 20.0 μg mL^{-1}, while the calibration curve for BPA included four concentrations from 0.5 to 10.0 μg mL^{-1}. The dilution solvent was methanol/water (20:80; v/v). The LOD and LOQ values of all substances are listed in Table 4. Calibration was validated by the distribution of data points on the residual plot and the coefficient of determination (r^2). A uniform residual plot with $r^2 \geq 0.95$ was taken as evidence for the linearity of the calibration. The calibration for all analytes was linear within the calibration range and, in all calibrations, $r^2 > 0.99$. Detection and quantification of Bis-GMA, UDMA, and TEGDMA were performed using HPLC-UV/Vis-MS. Due to low ionizability, BPA analysis was performed using HPLC coupled with ultraviolet–visible spectroscopy (HPLC-UV/Vis). To avoid false-positive results, all samples in which BPA was detected were prepared again and analyzed by HPLC-coupled tandem mass spectrometry (HPLC-MS/MS), using isotope-labeled BPA as the internal standard.

HPLC-MS/MS

Immediately before measurements, the eluates were thawed at room temperature. An aliquot of 1.5 mL was transferred from each eluate into HPLC amber glass vials and the eluates were dried using a speed vacuum concentrator (RVC 2–25 CD plus; Christ) at 37°C. The dry samples were derivatized and analyzed immediately. Bisphenol A was derivatized using pyridine-3-sulfonyl chloride, as described by Regueiro et al. [40]. The derivatization scheme is shown in Figure 1. Isotopically labeled BPA-d16 (d16BPA) was used as an internal standard. The chemicals used for derivatization and HPLC analysis are listed in Table 1.

The HPLC-MS/MS analysis was performed using an Agilent 1290 Infinity II HPLC system (Agilent Technologies) coupled to an Agilent 6460 triple quadrupole detector (Agilent Technologies). Separation was performed using a Polaris 3 C18-Ether column (100 × 2 mm with a 3 μm particle size; Agilent Technologies). The column was kept at 40°C, and the injection volume was 10 μL. Solvent A was water with 0.1% (v/v) formic acid, and solvent B was methanol with 0.1% (v/v) formic acid. The gradient was as follows: 0–0.2 min, 30% B; 0.2–6 min, 30%–98% B; 6–10 min, 98% B; 10–10.5 min, 98%–30% B; 10.5–14 min, 30% B. The eluent was ionized using an ESI source. Nebulizer pressure was 60 psi, and the capillary voltage was 4000 V. The inert gas was nitrogen at 350°C and the flow rate was 13 L min^{-1}. Bisphenol A and its derivatives were quantified in a multiple reactions monitoring (MRM) mode. The acquisition parameters are described in Table 5. All measurements were performed in duplicate.

The calibration curve of BPA consisted of 10 concentrations from 39 to 20,000 ng mL^{-1}, while the calibration curve for derivatized BPA (dBPA) included 16 concentrations from 0.005 to 156 ng mL^{-1}. The dilution solvent was acetonitrile/water (40:60; v/v). The LOD and LOQ values were calculated based on the standard deviation of the blank, as described by Wenzl et al. [41]. Calibration was validated as stated before. The calibration was linear within the calibration range and $r^2 > 0.99$. The LOD and LOQ values are shown in Table 4.

Statistics

Statistical analysis, including graphical processing, was performed using Microsoft Excel (Microsoft) and R version 3.6.1

TABLE 4 Limits of detection and analytical methods used for quantification of the monomers

Substance	Analytical method	Limit of detection	Limit of quantification
UDMA	HPLC-UV/Vis-MS	0.05 μg mL^{-1}	0.5 μg mL^{-1}
TEGDMA	HPLC- UV/Vis-MS	0.05 μg mL^{-1}	0.5 μg mL^{-1}
Bis-GMA	HPLC -UV/Vis-MS	0.5 μg mL^{-1}	–
BPA	HPLC-UV/Vis	0.5 μg mL^{-1}	0.5 μg mL^{-1}
dBPA	HPLC-MS/MS	0.009 ng mL^{-1}	0.03 ng mL^{-1}

Abbreviations: Bis-GMA, bisphenol A-glycidyl methacrylate; BPA, bisphenol A; dBPA, derivatized BPA; HPLC-MS/MS, high-performance liquid chromatography coupled with tandem mass spectrometry; HPLC-UV/Vis, high-performance liquid chromatography coupled with ultraviolet—visible spectroscopy; HPLC-UV/Vis-MS, high-performance liquid chromatography coupled with ultraviolet–visible spectroscopy and mass spectrometry; TEGDMA, triethylene glycol dimethacrylate; UDMA, urethane dimethacrylate.

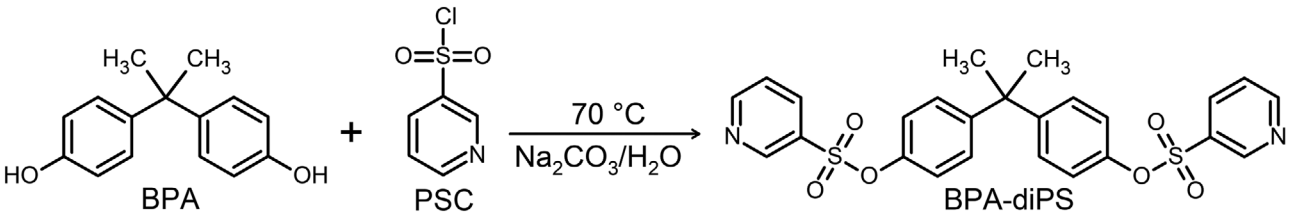

FIGURE 1 Derivatization of bisphenol A (BPA) with pyridine-3-sulfonyl chloride (PSC). BPA-diPS, bisphenol A derivatized with pyridine-3-sulfonyl chloride

TABLE 5 Acquisition parameters for bisphenol A (BPA) and its derivatives

Compound	Polarity	Parent ion	Product ions	Collision energy (V)	Fragmentor (V)	Cell accelerator voltage (V)
BPA	Negative	227	212	28	110	4
			133	28		
d16BPA	Negative	241	223	15	115	
			141	30		
BPA-diPS	Positive	511	354	35	163	
			290	35		
			276	30		
d16BPA-diPS	Positive	525	365	35	170	
			301	40		
			286	30		

Abbreviations: BPA-diPS, bisphenol A derivatized with pyridine-3-sulfonyl chloride; d16BPA, bisphenol A-d16; d16BPA-diPS, bisphenol A-d16 derivatized with pyridine-3-sulfonyl chloride.

(R Development Core Team). Statistical tests were used to determine significant differences between the materials after the period of maximal elution. Following a Shapiro–Wilk test to ensure normal distribution and a Levene's test to check for variance homogeneity, a one-way ANOVA followed by a Tukey post-hoc test was performed. The significance level was set to 0.05.

RESULTS

In the present investigation, the average concentrations of monomers eluted appeared to be material-dependent; in addition, a high degree of variability within the materials was observed, especially between BPA-free and conventional composites (see Table 6). Whereas UDMA and/or TEGDMA were detectable in the eluates of all materials after the period of maximum elution, Bis-GMA was not detected in any eluate after the period of maximum elution. In the eluates of two composites, BPA was detected and quantified using PDA. These results were not reproducible using HPLC-MS/MS and were therefore considered as false positive. A representative chromatogram of a sample in which UDMA and TEGDMA were detected is shown in Figure 2. The results for each C&B material are discussed in the remainder of this section.

TABLE 6 Concentrations of monomer released by different crown and bridge materials according to duration of incubation

Material	Incubation period	Bis-GMA (μg mL^{-1})	BPA (μg mL^{-1})	TEGDMA (μg mL^{-1})	UDMA (μg mL^{-1})
ExperTemp	1 h	–	–	<LOD	6.23 ± 0.8
	12 h	–	–	<LOD	6.74 ± 1.4
	24 h	<LOD	<LOQ$^{MS/MS}$	<LOQ	8.31 ± 1.6
	7 days	–	–	<LOD	5.46 ± 0.7
Luxatemp Automix Plus	1 h	–	–	<LOQ	<LOD
	12 h	–	–	1.34 ± 0.4	1.24 ± 0.4
	24 h	<LOD	<LOD$^{UV/Vis}$	1.03 ± 0.1	0.95 ± 0.1
	7 days	–	–	0.94 ± 0.2	1.08 ± 0.3
Protemp 4	1 h	–	–	<LOD	<LOD
	12 h	–	–	<LOD	<LOD
	24 h	<LOD	<LOD$^{UV/Vis}$	<LOQ	<LOD
	7 days	–	–	<LOD	<LOD
Visalys Temp	1 h	–	–	<LOD	1.18 ± 0.4
	12 h	–	–	<LOD	3.35 ± 1.2
	24 h	<LOD	<LOQ$^{MS/MS}$	<LOD	3.45 ± 1.1
	7 days	–	–	<LOD	4.11 ± 0.5

Values are given as mean ± SD.

Abbreviations: Bis-GMA, bisphenol A-glycidyl methacrylate; BPA, bisphenol A; LOD, limit of detection; LOQ, limit of quantification; MS/MS, tandem mass spectrometry; TEGDMA, triethylene glycol dimethacrylate; UDMA, urethane dimethacrylate; UV/Vis, ultraviolet–visible spectroscopy.

LOD: UDMA and TEGDMA, 0.05 μg mL^{-1}; Bis-GMA, 0.5 μg mL^{-1}.

LOQ: UDMA and TEGDMA, 0.5 μg mL^{-1}.

LOD$^{UV/Vis}$ BPA, 0.5 μg mL^{-1}.

LOQ$^{MS/MS}$ BPA, 0.00003 μg mL^{-1}.

Conventional C&B composites

Neither Bis-GMA nor BPA was detected in the eluates of Protemp 4 and Luxatemp Automix Plus. Moreover, UDMA was not detectable in the eluates of Protemp 4, and TEGDMA levels were below the LOQ after the period of maximum elution (24 h of incubation). Quantifiable amounts of UDMA and TEGDMA were eluted from Luxatemp Automix Plus, especially in the first 12–24 h of incubation.

BPA-free C&B composites

No Bis-GMA was detectable in the eluates of Visalys Temp and ExperTemp. Quantifiable amounts of BPA were detected in the eluates of both Visalys Temp and ExperTemp by HPLC-UV/Vis. After 24 h of incubation, 3.1 ± 0.18 μg mL^{-1} of BPA was detected in eluates of ExperTemp and 1.6±0.22 μg mL^{-1} in eluates of Visalys Temp. Due to the well-known false-positive results of this detection technique [42–45], all samples were reanalyzed. Using tandem mass spectrometry, the levels of BPA previously measured by HPLC-UV/Vis were not reproducible, and these results were therefore considered as false positive. After 24 hours of Incubation no TEGDMA was detectable in Visalys Temp eluates and the concentrations were below the LOQ in ExperTemp eluates. Quantifiable amounts of UDMA were detectable in the eluates of both Visalys Temp and ExperTemp, with most of the release occurring within the first 24 h of incubation.

Statistical analysis was performed solely for UDMA because only this monomer was quantifiable in the eluates of more than one material (see Figure 3). As UDMA was not detectable in the eluates of Protemp 4, it was therefore assumed that UDMA was not released from this material, which was therefore given 0-values in the following statistical evaluation. The Shapiro–Wilk test showed a normal distribution of data across all groups (ExperTemp $P = 0.313$; Luxatemp $P = 0.06321$; Visalys Temp $P = 0.163$). Variance homogeneity could not be refuted by a Levene test ($F = 1.7295$, $P = 0.22$). An ANOVA revealed highly significant differences between the C&B materials regarding UDMA release ($P < 0.001$). Tukey's post-hoc test showed significant differences between the materials studied. The amounts of UDMA released were significantly higher in ExperTemp eluates than in all other materials (Visalys Temp < 0.001; Luxatemp Automix Plus $P < 0.001$; Protemp 4 $P < 0.001$). Visalys Temp eluted significantly more UDMA than Protemp 4 ($P < 0.001$) and Luxatemp Automix Plus

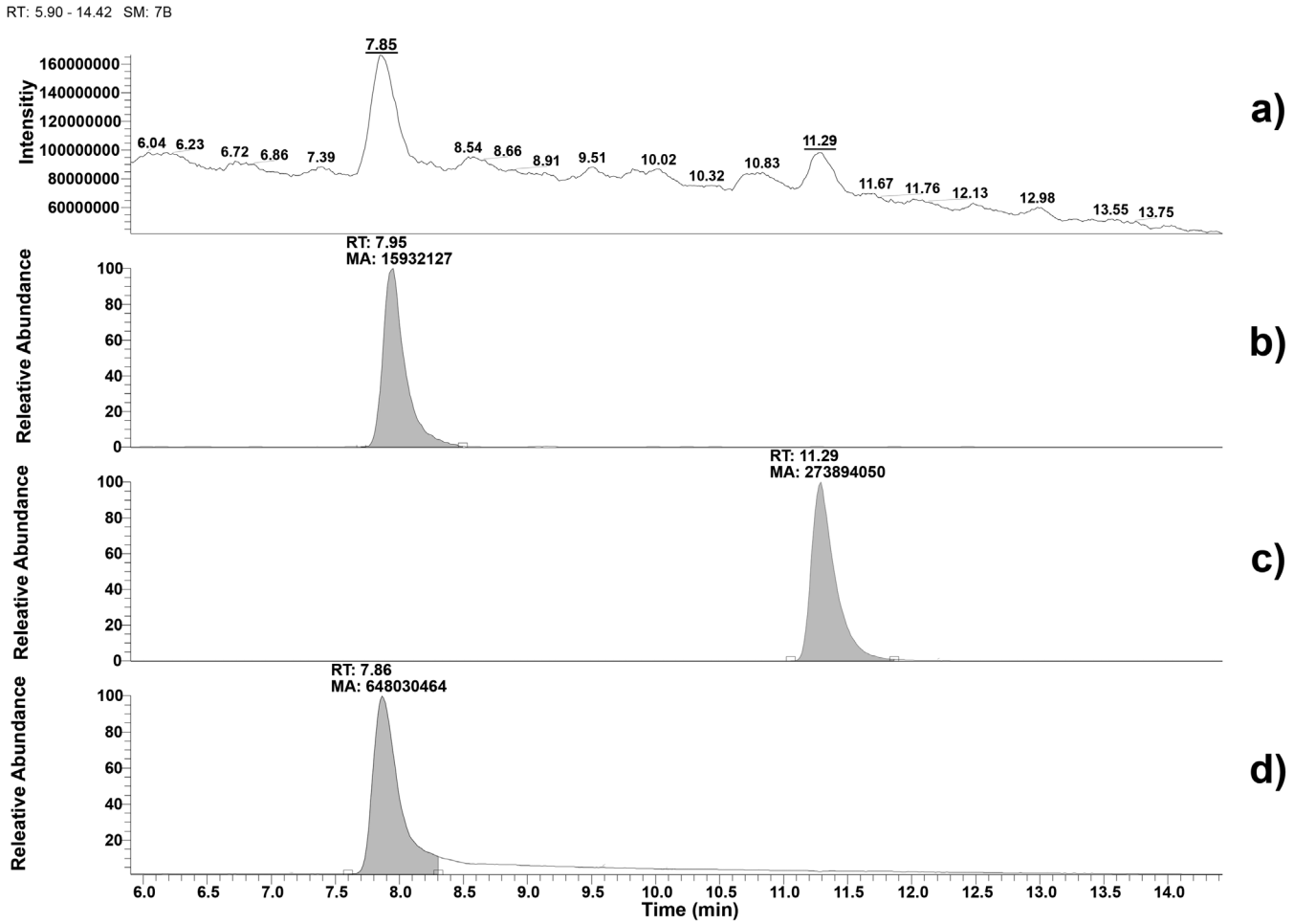

FIGURE 2 Chromatogram of an ExperTemp sample obtained after an incubation period of 24 h. (a) Chromatogram: peaks identified by retention time are underlined. (b) Relative abundance corresponding to triethylene glycol dimethacrylate (TEGDMA). (c) Relative abundance corresponding to urethane dimethacrylate (UDMA). (d) Relative abundance corresponding to diethyl phthalate (internal standard); RT: retention time, MA: manual area under peak

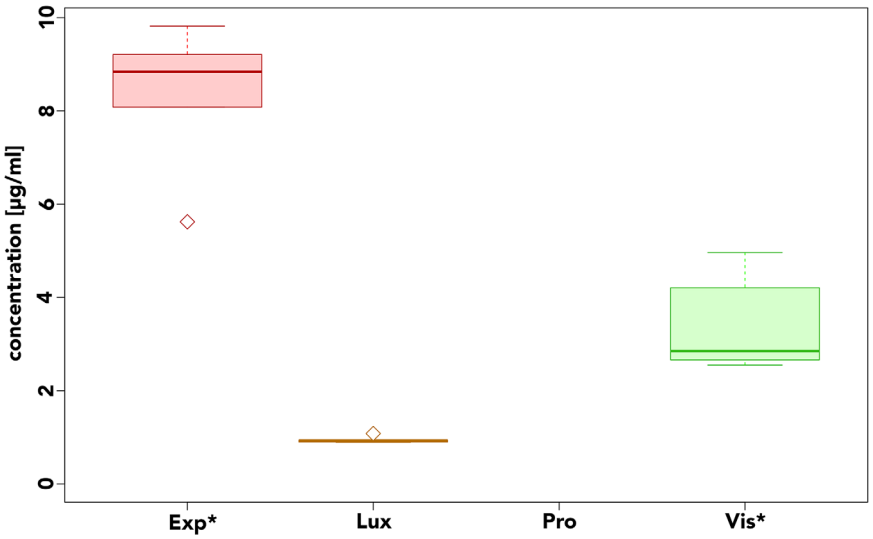

FIGURE 3 Urethane dimethacrylate (UDMA) release, after 24 h of incubation, from each crown and bridge (C&B) material investigated. Exp, ExperTemp; Lux, Luxatemp Automix Plus; Pro, Protemp 4; Vis, Visalys Temp; *Bisphenol A (BPA)-free

($P = 0.005$), although the difference between the latter was not significant ($P = 0.44$). In summary, a larger amount of UDMA was released from BPA-free materials than from conventional C&B composites.

DISCUSSION

In the present study, the elution of BPA and the three most relevant monomers from four different C&B composite materials was investigated over a 7-day period. The monomers eluted, and their quantity, varied considerably according to the C&B material analyzed. It was shown that significantly more UDMA was eluted from BPA-free materials in aqueous media than from conventional composites containing BPA derivatives. In general, our data show that, after 24 h, almost all monomers were completely released. This is already known from other studies on different composite materials [29,46–48]. For these reasons, the 24-h incubation period was defined as the period of maximum elution in this study. Based on other studies, we hypothesized that Bis-GMA and BPA are either not released or released only in small quantities in aqueous media, such as water, artificial saliva, or collected saliva [26–28]. For detection and exclusion of Bis-GMA and BPA release, we analyzed the eluates after the period of maximum elution because this incubation period is considered the reference time for meta-analysis [25]. After this period of incubation, Bis-GMA was not detectable, regardless of the C&B material analyzed. By contrast, BPA was detected and quantified in the eluates from ExperTemp and Visalys Temp using HPLC-UV/Vis. This was a highly unusual finding as both ExperTemp and Visalys Temp are labeled as BPA-free. However, it is known from various scientific fields that detection based solely on UV/Vis can lead to false-positive results; thus, misidentification of co-eluting substances must be taken into account [42–44]. Hope et al. [45] found that the misidentification of a co-eluting compound which shows similar chemical characteristics to a photoinitiator used in dental resins can lead to the overestimation of BPA levels by 30-fold. Therefore, all samples in which BPA was detected were re-analyzed. The control analysis was performed by HPLC-MS/MS calibrated with d16BPA as the internal standard. This analytical method is well established in recent literature [49–51] and is recommended by Hope et al. [45]. The concentrations measured using HPLC-UV/Vis could not be reproduced as the amount of BPA detected was below the LOQ of 0.03 ng mL^{-1}. It is known from analytical chemistry that BPA concentrations in the low nanogram range are often the result of contamination, even in analytical laboratories, resulting from the ubiquitous use of BPA [52]. The authors of this study took strict precautions to avoid contamination and used materials made from borosilicate glass or PTFE, both of which are considered BPA-free [53–56]. However, previous studies detected BPA concentrations in the low nanogram range, even in pure HPLC-grade water [57–59]. As the signal strength was identical across materials and in the low nanogram range, the concentrations of BPA detected in the present study are probably a result of the ubiquitous use of BPA. The false-positive results obtained support the claim of Hope et al. [45] that mass spectrometry is the preferred method for detection and quantification of BPA released from dental resins, and that use of UV/Vis for such purposes should only be performed with great caution.

We detected TEGDMA and/or UDMA in the eluates of all materials, after at least one incubation period, and BPA-free materials seemed to elute significantly more UDMA than conventional materials. By using the extraction ratio given in ISO 10993-12, we were able to assess the clinical significance of monomer elution by relating the concentrations of monomers eluted to common sizes of restoration (see Table 7). The cytotoxic effective concentration of dental monomers is expressed as the TC_{50} concentration [60, 61]. It is usually determined after 24 h or 48 h of exposure of different cell lines to the respective monomer [62, 63]. The TC_{50} concentrations of UDMA for human gingival fibroblasts, after 24 h of exposure, have been reported to range between 49.88 (± 2.35) and 94.11 (± 47.06) $\mu g\ mL^{-1}$ [64–66]. The cytotoxic concentrations of TEGDMA are significantly higher, ranging between 509.65 (± 37.22) and 1033.61 (± 57.26) $\mu g\ mL^{-1}$ [64–67]. Comparison between the concentrations of monomers measured (Table 6 and 7) and the cytotoxic-effective monomer concentrations described indicates that cytotoxic effects from restorations are unlikely, even with larger temporary restorations. Regarding the synergistic effect of UDMA plus TEGDMA, contradictory statements on the combined effect of UDMA and TEGDMA can be found in the literature [68–70]. Nevertheless, even the highest concentrations of monomers measured are below the lowest described combined synergistic TC_{50} concentration [69]. As a result of salivary flow and intraoral degradation by abrasion, erosion, and enzymes within saliva, the concentrations of monomers released in vivo might be higher than those released in vitro [71]. In cytotoxicity tests, however, these dynamics are often not taken into account [72]. Usually the constant salivary flow is not considered because a standing, non-renewing cell culture medium is often used [63,73]. However, in vivo, monomers released are cleared early and therefore the concentrations measured in vitro can only be reached and maintained in confined spaces, such as deep cavities [74]. Thus, it seems unlikely that the materials investigated in the present study release monomers at the respective TC_{50} concentrations, even when used for large temporary restorations.

Besides cytotoxicity, the mutagenic effects of dental monomers on local cells are discussed in the current literature.

TABLE 7 Average concentration of monomers released from the most common types of restoration (after 24 h of incubation)

Material	Restoration	TEGDMA ($\mu g\ mL^{-1}$)	UDMA ($\mu g\ mL^{-1}$)
ExperTemp	Crown (first molar)	<LOQ	5.56
	Bridge (second premolar to second molar)	<LOQ	12.91
Luxatemp Automix Plus	Crown (first molar)	0.69	0.64
	Bridge (second premolar to second molar)	1.60	1.48
Protemp 4	Crown (first molar)	<LOQ	–
	Bridge (second premolar to second molar)	<LOQ	–
Visalys Temp	Crown (first molar)	–	2.31
	Bridge (second premolar to second molar)	–	5.36

Surface areas (crown = 3.15 cm^2; bridge = 7.32 cm^2) were calculated in accordance with Van Landuyt et al. [25]

Abbreviations: LOD, limit of detection; LOQ, limit of quantification; TEGDMA, triethylene glycol dimethacrylate; UDMA, urethane dimethacrylate.

LOD: UDMA and TEGDMA, 0.05 $\mu g\ mL^{-1}$.

LOQ: UDMA and TEGDMA, 0.5 $\mu g\ mL^{-1}$.

These effects have been demonstrated for TEGDMA and UDMA on various cell lines [75–78] and to occur even below the respective TC_{50} concentration [79–81]. Concentrations at which mutagenic effects (such as double-strand breaks, deletions of DNA segments, or the induction of micronuclei) occur, depend on the type of cell exposed and the dental monomer investigated [82,83]. In human gingival fibroblasts, double-strand breaks were observed at TEGDMA concentrations of 103.08 $\mu g\ mL^{-1}$ after 24 h of exposure [84]. After the same period of exposure, clastogenic effects were detected on V79 hamster cells at TEGMDA concentrations of 71.58 $\mu g\ mL^{-1}$ [85]. In human lymphocytes, DNA damage occurs at TEGDMA concentrations as low as 2.86 $\mu g\ mL^{-1}$ after only 60 min of exposure [76]. In the present study, the materials investigated did not release TEGDMA concentrations as high as the mutagenic TEGDMA concentrations described in the literature. Even in large temporary restorations, these concentrations are not to be expected.

By contrast, UDMA exhibits a mutagenic effect that is more potent by a factor of 1.6 [86,87]. Using a comet assay with human parotid gland cells and lymphocytes, initial DNA damage was observed after only 60 min of exposure at a UDMA concentration of 0.047 $\mu g\ mL^{-1}$ [75,76]. After 6 h of exposure, a relevant number of double-strand breaks in human gingival fibroblasts was detected at a UDMA concentration of 14.12 $\mu g\ mL^{-1}$ [84]. These genotoxic effects were confirmed in vivo using eluates of UDMA- and/or TEGDMA-based composites [86]. By contrast, an older study by Schweikl et al. [88] found only a slight increase of micronuclei compared with the control group after exposure to UDMA. In the present study, quantifiable UDMA concentrations were detected in Luxatemp Automix Plus, ExperTemp, and Visalys Temp samples. Depending on the restoration size and the material used, release of between 0.64 and 12.91 $\mu g\ mL^{-1}$ UDMA can be expected after 24 h (see Table 7). As mutagenic effects of UDMA have been observed for short exposure times and at low concentrations, mutagenic effects of the materials investigated in the present study cannot be excluded. For further clarification, in vitro or in vivo studies with eluates of the investigated materials are required.

This study is not without limitations. The major difficulty of in vitro studies is consideration of all influencing factors of the oral environment [61] and additionally, in the authors' experience, the fabrication of temporary restorations varies greatly in daily practice. Earlier studies found that monomer elution is significantly influenced by the procedure chosen to remove the oxygen inhibition layer (polishing/ethanol treatment/no treatment) [89,90] and, to our knowledge, the effect of different varnishes for temporary restorations on monomer elution has not been investigated. However, the authors of this study suspect that the method used to remove the oxygen inhibition layer and the use of a varnish significantly influence the elution of monomers. Immediate immersion after setting of the samples might also have influenced monomer elution. Some studies performed immersion after the post-irradiation cure, usually a storage period of 24 h in the dark [33,45,91–93]. This procedure leads to reduced release of monomer [94] but is not consistent with the clinical workflow [33,95]. Therefore, the authors decided against this procedure. When interpreting the present results, it should be taken into account that different clinical workflows probably influence the amount of monomer released.

During incubation, agitation is required to simulate the salivary flow and to reduce contact of the samples with the floor of the vial to ensure standardized extraction [25]. In current literature, agitation is not standardized and varies between 20 and 200 rpm [93,96–98]. In preliminary tests, we found a significant reduction of vial contact at 112 rpm, which assures standardized extraction. We observed decreasing monomer concentration in successive incubation periods. This is a common finding in studies in which each incubation period was analyzed separately [27,28,48,93], as opposed to studies that

measured elution cumulatively [29,46, 71]. Presumably, passive hydrolysis reactions lead to the degradation of monomers in water [99]. This and most other studies have not performed a solvent refresh [25]. Therefore, water sorption processes, which can lead to a change of the extraction ratio [100], can be another reason for this observation. However, in other extraction media, such as ethanol/water mixtures or lactic acid, this observation was either not made or was made to a lesser extent [27,48]. Passive and/or enzyme-catalyzed hydrolysis, such as in collected saliva, breaks the ester bonds of the methacrylate groups of Bis-GMA, TEGDMA, and UDMA [101–104]. The hydrolysis of dental monomers is often incomplete, so that molecules with a different number of cleaved methacrylate groups may be present at the same time [103,105]. These hydrolysis products each have different chemical properties and molar masses, so detection requires adjustment of the analytical method [105,106]. Another limitation of this and many other studies is the selection of reference substances, as the manufacturers are not obliged to disclose the full composition of their materials because this is considered a trade secret [97,107]. In the present study, analysis was performed of the most common monomers and BPA [9,108,109] but not of the BPA derivative, bisphenol A ethoxylated dimethacrylate (Bis-EMA). Manufacturers of composite materials use Bis-EMA molecules with varying degrees of ethoxylation [110]. The required adaptation of the analytical method is very costly as well as time-consuming and therefore was not possible in this study. Consequently, the elution of Bis-EMA cannot be ruled out and a follow-up study is needed to evaluate the release of Bis-EMA and degradation products of dental monomers.

Within the limitations of this study, it is concluded that monomer elution from the investigated materials is material-dependent, but release of BPA or cytotoxic effective concentrations of the investigated monomers is unlikely. Most biocompatibility concerns about the use of dental composites are related to the release of BPA and its derivatives, such as Bis-GMA. However, none of these substances was detectable in the present study. Furthermore, the present study showed that detection of BPA solely by HPLC-UV/Vis may lead to false-positive results. As this was also shown in a previous study [45], we recommend verifying HPLC-UV/Vis results by mass spectrometry. In BPA-free composites, BPA derivatives are usually replaced by UDMA. These adjustments to the polymer matrix resulted in a significant increase of UDMA concentrations measured in the present investigation. Therefore, the replacement of BPA derivatives with UDMA, in terms of biocompatibility, should be reconsidered. However, all concentrations of monomer were below the reported cytotoxic-effective monomer concentrations. Nevertheless, we strongly recommend adhering strictly to the manufacturer's instructions. In particular, cleaning with alcohol should be carried out, as significant turbidity of this cleaning agent was noticed in the course of this study.

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interests.

AUTHOR CONTRIBUTIONS

Conceptualization: T. Hampe, S. Krohn, R. Bürgers; **Methodology**: T. Hampe, A. Wiessner, R. Bürgers, S. Krohn; **Investigation**: T. Hampe, A. Wiessner, H. Frauendorf, M. Alhussein, P. Karlovsky; **Formal analysis**: T. Hampe; **Writing – review and editing**: T. Hampe, A. Wiessner, R. Bürgers, S. Krohn, H. Frauendorf, M. Alhussein, P. Karlovsky; **Visualization**: T. Hampe, M. Alhussein, P. Karlovsky; **Supervision**: T. Hampe, S. Krohn; **Project administration**: S. Krohn, R. Bürgers; All authors approved the final version of this article.

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