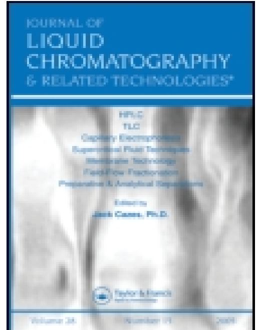

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DEVELOPMENT AND VALIDATION OF AN ISOCRATIC HPLC METHOD FOR THE SIMULTANEOUS DETERMINATION OF RESIDUAL MONOMERS RELEASED FROM DENTAL POLYMERIC MATERIALS IN ARTIFICIAL SALIVA

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□ *A simple and rapid method for the simultaneous determination of Bisphenol-A (BPA), Triethylene glycol dimethacrylate (TEGDMA), Bisphenol A glycerolate dimethacrylate (BIS-GMA), and Urethane dimethacrylate (UDMA) from dental polymeric materials in artificial saliva is described. Chromatographic analysis was performed isocratically on a Kromasil 100-C_{18} analytical column (25 cm × 4.6 mm id, 5 μm) with CH_3CN:H_2O, 75:25%, v/v as mobile phase within 6 min. The developed method was validated in terms of selectivity, linearity, accuracy, precision, and sensitivity. Accuracy and precision were examined at three concentration levels. Repeatability and between-day precision was examined over a period of five days and revealed RSD values lower than 11.2%. The relative errors ranged from −13.8% to 3.9%. In artificial saliva the limit of quantification (LOQ) was calculated as 1.2–3.6 ng/μL. No interference was observed under described experimental conditions. Residual monomers released from dental materials were quantified after exposure of specimens to different solvent ratios.*

Keywords artificial saliva, bisphenol-A (BPA), bisphenol A glycerolate dimethacrylate (BIS-GMA), dental composites, dimethacrylate monomers, HPLC, triethylene glycol dimethacrylate (TEGDMA), urethane dimethacrylate (UDMA)

INTRODUCTION

Dental composites are tooth filling materials and restorative materials. The chemical composition of dental composites is complicated as they consist of synthetic polymers, ceramic reinforcing fillers, molecules that

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promote or modify the polymerization reaction that yields the cross-linked polymer matrix from the dimethacrylate resin monomers, and silane coupling agents that enable bonding of the reinforcing fillers to the polymer matrix. Composites without inorganic fillers are known as "sealants." Each of these components is critical for a successful dental restoration. A comprehensive review of the composition and characteristics of current dental composites can be found in literature written by Ferracane.[1]

Methacrylate compounds are widely used in restorative dentistry as composite resin restorations, and they are considered as substitutes to amalgam fillings, as sealants, bonding agents, and resin cements. The use of resin-based dental restorative materials is rapidly increasing since they have excellent mechanical properties, rapid polymerization, aesthetic quality, and the ability to bond to enamel surface.[1,2]

Different substances that are released from many dental resin composites have been under investigation by various researchers. Geurtsen et al. have investigated the composition and cytotoxicity of aqueous extracts of four light-curing pit and fissure sealants. These researchers have found that some of these substances presented in several in vitro studies cytotoxic, genotoxic, mutagenic, or estrogenic effects and pulpal and gingival mucosa reactions.[3,4]

The most commonly used monomers for the preparation of dental resins are crosslinking dimethacrylates, such as bisphenol A glycol dimethacrylate (BIS-GMA), Triethylene glycol dimethacrylate (TEGDMA), and Urethane dimethacrylate (UDMA). Mixtures of these monomers are generally used. The choice of the monomers influences, to a great extent, the reactivity, viscosity, and polymerization shrinkage of the monomer, as well as the mechanical properties, water uptake, and the swelling by water of the resin. Acrylates and methacrylates that had been used in dental resin materials showed a relationship between their structure and the degree of cytotoxicity. TEGDMA and, above all, BIS-GMA and UDMA showed high cytotoxicity.[5]

Concerning Bisphenol A (BPA), in principle, this compound is not a component of dental resin composite, but it may be present as an impurity in some resins. Many epoxy resins and polycarbonates are synthesized from BPA. Other sources of BPA are contaminants in wine and mineral water stored in plastic containers in microwave processors, in food from cans coated with epoxy resin lacquers, and in canned oily foods. Recently, researchers found that BPA leached into saliva of treated patients was responsible for the estrogenicity of some commercial composites and sealants used in dentistry.[3,4] Although BPA has low acute toxicity, with an oral LD_{50} of 3250 mg/kg in rats, its harmful effect arises from the fact that it is an endocrine disruptor. Low doses of Bisphenol A can mimic the body's own hormones, possibly causing negative health effects. Therefore, there is high concern that long term low dose exposure to Bisphenol A may induce chronic toxicity in humans.[2]

The elution process of monomers released from dental resin composites has been extensively studied in the literature concerning the quantity of leachable monomers and the time needed for the complete elution. These parameters are affected by three factors: (a) the amount of unreacted monomers, which is determined by the chemical structure of the monomer and the polymerization conditions; (b) the solvent used for the elution and the elution conditions; and (c) the size and the chemical structure characteristics of the monomers. It is presumed that small molecules like TEGDMA have enhanced mobility and will be eluted faster than larger molecules like BIS-GMA.[6,7]

As TEGDMA, BIS-GMA, and UDMA differ widely in their volatility, due to their different chemical structure, as shown in Figure 1, and in their

Compounds	Chemical structures	Molecular type
Bisphenol-A (BPA)		$C_{15}H_{16}O_2$
Triethylene glycol dimethacrylate (TEGDMA)		$C_{14}H_{22}O_6$
Urethane dimethacrylate (UDMA)		$C_{23}H_{38}N_2O_8$
Bisphenol A glycerolate dimethacrylate (BIS-GMA)		$C_{29}H_{36}O_8$

FIGURE 1 Chemical structures and molecular types of examined analytes.

stability in saliva, a wide variety of analytical methods have been developed for each monomer individually or for more compounds simultaneously using different analytical techniques, for their quantitative determination into saliva, or for their identification and quantification after their release from dental materials.[2]

Due to the complexity of the matrix and the fact that different by-products may be present, a separation technique is mandatory for the study of residual monomers in saliva. According to our review of recent literature, HPLC is generally used for the quantitative and qualitative determination of the residual monomers eluted from light-cured dental resins and resin composites.[5,8–11] Already published methods are not validated and most of them suffer by resolution problems. The identification of the released components is usually performed by utilizing liquid chromatography/mass spectrometry and gas chromatography.[12–19] Yet, it is necessary to note that monomers of high molecular weight such as BIS-GMA ($MW = 512$) and UDMA ($MW = 470$) decompose during processing in a gas chromatograph; therefore, only their decomposition products can be detected. Volatile materials have been analyzed by gas chromatography/mass spectrometry (GC/MS), while less volatile materials have been separated by liquid chromatography (LC).[20–22]

The aim of this study was to develop and validate a simple, fast, accurate, and precise HPLC method for the simultaneous determination of BPA, TEGDMA, UDMA, and BIS-GMA from dental polymeric materials in artificial saliva. The applicability of the method was evaluated by studying the amounts of monomers released from polymeric dental materials that were immersed in different solvents at different volume ratios.

EXPERIMENTAL

Chemicals

Methanol and acetonitrile of HPLC grade were supplied by Fisher Scientific Limited (Bishop Meadow Road, UK). Ethanol p.a. was from Merck KGaA (Darmstadt, Germany). Ultrapure water was obtained by using a Milli-Q ultrapure water system (Millipore, Bedford, MA, USA). BPA, TEGDMA, UDMA, and BIS-GMA were of analytical grade and obtained from Sigma-Aldrich (Steinheim, Germany). Individual stock solutions were prepared by dissolving an appropriate amount of each substance in methanol or artificial saliva and found to be stable for at least five months. For artificial saliva, sodium bicarbonate ($NaHCO_3$), sodium chloride (NaCl), potassium dihydrogen phosphate (KH_2PO_4), and disodium hydrogen phosphate (Na_2HPO_4) from Merck; citric acid monohydrate ($C_6H_8O_7 \cdot H_2O$) from (Riedel-de Haen, AG D-3016, Seelze 1, Germany); and calcium chloride ($CaCl_2$) were used.

Instrumentation and Chromatographic Conditions

An LC–10_{AD} pump by Shimadzu (Kyoto, Japan) was used to deliver mobile phase to the analytical column. Sample was injected via a Rheodyne 7125 injection valve (Rheodyne, Cotati, California, USA). Detection was achieved at a wavelength of 230 nm and sensitivity setting of 0.002 AUFS using an SSI 500 UV-vis detector (SSI, State College, PA, USA). The software used for data acquisition was developed by Professor P. Nikitas (Laboratory of Physical Chemistry, Chemistry Department of University of Thessaloniki).

Milli-Q water used for mobile phase was filtered using a glass vacuum-filtration apparatus, obtained from Alltech Associates, (Deerfield, IL, USA) and Whatman Cellulose Nitrate 0.2 μm – WCN Type (47 mm DIA) membrane filters (Whatman Laboratory Division, Maidstone, England). Degassing of solvents was achieved by helium sparging prior to use.

A Kromasil 100-C_{18} analytical column (25 cm × 4.6 mm id, 5 μm) purchased from HICHROM (Berkshire, UK) was used for the separation at ambient temperature. Mobile phase consisted of acetonitrile-water (75:25%, v/v) was delivered isocratically at a flow rate of 1.0 mL/min.

Preparation of Standards

Stock solutions of 360, 100, 300, and 500 ng/μL of BIS-GMA, UDMA, BPA, and TEGDMA, respectively, were prepared by dissolving the appropriate amount of the each residual monomer in 50 mL of methanol. Methanolic working standards were prepared in the range of 0.2–20 ng/μL for all residual monomers. Eight calibration points were used at concentrations 0.2, 0.5, 1, 2, 5, 10, 15, and 20 ng/μL.

Solutions were stored refrigerated. Calibration levels of BIS-GMA, UDMA, BPA, and TEGDMA in artificial saliva were prepared by diluting the stock solution with artificial saliva.

Preparation of Specimens

The specimens were prepared according to the manufacturer's instructions. Cylindrical teflon moulds were filled with uncured material (3M ESPE, Filtek Supreme XT, A_2 Body Shade, USA) to produce specimens with a diameter of 7 mm and a thickness of 2 mm. The uncured material was covered with a polyester film (matrix strip DIRECTA) and a glass plate to hinder the development of the oxygen-inhibiting layer, and was irradiated by visible light-curing unit (Elipar trilight 3M ESPE) of 850 mW cm-2 for 40 s. The light intensity of the unit was regularly controlled by a Helux Curing Light Meter (BENLIOGLU DENTAL INC (USA). The unit was used without the light guide, at a distance from the sample 1 mm. After polymerization, the

specimens were immersed in 25 mL of mixtures of ethanol with water and ethanol with artificial saliva solutions in different volume ratios: 75:25, 50:50, and 25:75%, v/v. Glass beakers containing specimen and solvent were stored in an oven at 37°C and they were left for 24 hr, 7 d and 14 d. The samples were agitated from time to time and mixed prior to sampling for analysis.

Then, aliquots of 20 μL were taken for HPLC analysis. Three specimen discs were prepared for each mixture. Artificial saliva (pH range 6–7) consisted of 7.5 mM KH_2PO_4, 7.5 mM Na_2HPO_4, 15 mM $NaHCO_3$, 1.5 mM $CaCl_2$, 0.9 mM $C_6H_8O_7 \cdot H_2O$, and 10 mM NaCl in ultra-pure water.[23]

Method Validation

The developed method was validated in terms of selectivity, linearity, accuracy, precision, and sensitivity. The linearity of the analytical method was studied using mixtures of methanolic standard solutions covering the entire working range. Calibration curves with respective correlation coefficients, slopes, and intercepts resulted from the linear regression analysis.

Selectivity was studied by assessing the absence of interference in the same chromatographic windows as the examined BIS-GMA, UDMA, BPA, and TEGDMA in artificial saliva. Analysis of blank matrices was used to demonstrate selectivity.

Accuracy and precision were examined at three concentration levels. Accuracy was expressed as relative error in artificial saliva samples. Linearity was studied by constructing the calibration curves using stock solutions and stock solutions with pure artificial saliva at concentration levels in the range of 1.2–20 ng/μL. Calibration lines were constructed from peak areas of analytes. The calculation of the slope, the intercept, and the correlation coefficient of each calibration line was achieved through linear regression analysis.

Repeatability and between-day precision over a period of five days revealed Relative Standard Deviation (RSD) values lower than 11.2%. The relative errors ranged from −13.8 to 3.9%. Limit of Detection (LOD) values were calculated from the calibration curve according to the formula $LOD = 3.3\,s/S$ and Limit of Quantification (LOQ) values according to the formula $LOQ = 10\,s/S$, where S = the slope and s = standard deviation of intercept. Precision expressed by RSD was checked at three concentration levels.

RESULTS AND DISCUSSION

Chromatography

Quantitative analysis was performed on a HPLC system with UV-VIS detector (230 nm). The separation of residual monomers BIS-GMA, UDMA, TEGDMA and BPA was achieved within 6 min. Resolution factors ranged

from 2.4 to 3.6 indicating an appropriate separation. Retention times for the examined compounds were 3.2 min for BPA, 4.4 min for TEGDMA, 5.4 min for UDMA, and 5.9 for BIS-GMA. A typical chromatogram of a standard solution is shown in Figure 2a.

Calibration curves were drawn for each standard material at different concentrations.

Validation Method

Linearity

The upper limit of linear range for methanolic standard solutions and spiked artificial saliva was 20 ng/μL for all monomers except for TEGDMA, which was linear up to 15 ng/μL. The responses versus nominal concentrations fitted well to a straight line. Correlation coefficients ranged from 0.9990 to 0.9963 for standard solutions and for saliva from 0.9996 to 0.9970. In artificial saliva samples LOQ was calculated as 1.2–3.6 ng/μL. Linearity and sensitivity results are shown in Table 1.

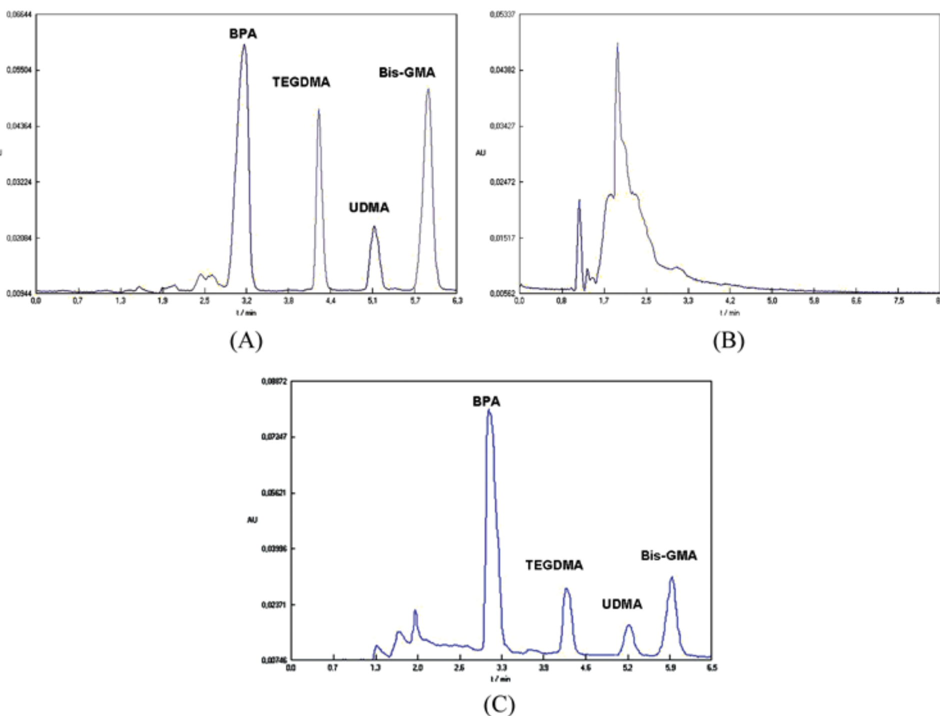

FIGURE 2 Typical HPLC chromatogram of the BPA, TEGDMA, UDMA, and BIS-GMA in: A) Methanolic standard at 5 ng/μL; B) blank artificial saliva; and C) spiked saliva solutions at 5 ng/μL. (Color figure available online.)

TABLE 1 Calibration and Sensitivity Data of BPA, TEGDMA, UDMA, and BIS-GMA in Standard Methanol and Artificial Saliva

Analyte	Regression Data	R	LOD (ng/μL)	LOQ (ng/μL)
	Standards			
BPA	Y = (0.1256 ± 0.0048)X − (0.0164 ± 0.0504)	0.9963	1.3	4.0
TEGDMA	Y = (0.0406 ± 0.0010)X + (0.0084 ± 0.0113)	0.9981	0.9	2.8
UDMA	Y = (0.0204 ± 0.001)X + (0.0011 ± 0.0066)	0.9965	1.1	3.2
BIS-GMA	Y = (0.0772 ± 0.0015)X + (0.0094 ± 0.0154)	0.9990	0.6	2.0
	Artificial Saliva			
BPA	Y = (0.1371 ± 0.0035)X + (0.0380 ± 0.0373)	0.9982	0.9	2.7
TEGDMA	Y = (0.035 ± 0.0005)X + (0.0143 ± 0.0142)	0.9996	0.5	1.5
UDMA	Y = (0.0177 ± 0.0002)X + (0.0122 ± 0.002)	0.9996	0.4	1.2
BIS-GMA	Y = (0.0645 ± 0.0025)X + (0.0149 ± 0.0234)	0.9970	1.2	3.6

Y = Peak area.
X = ng/μL.

Accuracy and Precision

Accuracy and precision varied between −13.8% to 3.9%. Repeatability and between-day precision over a period of five days revealed RSD values lower than 11.2%. Results are summarized in Table 2.

Selectivity

The absence of interference in the same chromatographic window proved the selectivity of the method. Typical chromatograms of blank and spiked saliva samples are illustrated in Figure 2B and 2C respectively.

TABLE 2 Within-Day Repeatability, Between-Day Precision and Accuracy for the Determination of BPA, TEGDMA, UDMA, and BIS-GMA in Artificial Saliva

		Within-Day ($n = 5$)			Between-day ($n = 5$)		
Analyte	Added (ng)	Found ± SD (ng)	RSD	Relative Error %	Found ± SD (ng)	RSD	Relative Error %
BPA	20	18.6 ± 0.02	0.1	−7.0	20.7 ± 1.4	6.7	3.5
	100	103.5 ± 0.11	0.1	3.5	103.9 ± 2.8	2.7	3.9
	200	180.1 ± 0.20	0.1	−9.9	187.1 ± 6.1	3.3	−6.4
TEGDMA	20	20.7 ± 0.11	0.5	3.5	20.4 ± 2.2	10.8	2.0
	100	92.9 ± 0.23	0.3	−7.1	102.5 ± 8.6	8.4	2.5
	200	181.7 ± 0.18	0.1	−9.1	183.4 ± 10.5	5.7	−8.3
UDMA	20	18.5 ± 0.15	0.8	−7.5	20.5 ± 2.3	11.2	2.5
	100	97.3 ± 6.30	6.5	−2.7	96.0 ± 2.4	2.5	−4.0
	200	187.5 ± 0.17	0.1	6.2	175.9 ± 9.1	5.2	−12.0
BIS-GMA	20	20.2 ± 0.32	1.6	1.0	20.2 ± 0.4	2.0	1.0
	100	86.2 ± 0.09	0.1	−13.8	89.1 ± 0.4	0.5	−10.9
	200	205.5 ± 0.10	0.05	2.8	185.9 ± 20.3	10.9	−7.0

Method Application

The applicability of the method was tested in a study of the residual monomers BPA, TEGDMA, UDMA, and BIS-GMA which can be released from polymeric dental materials and immersed in ethanol:water and ethanol:artificial saliva mixtures at different volume ratios.

Specimen discs were prepared as described in the Experimental section. Then, the specimens were immersed in mixtures of ethanol:water, and ethanol:artificial saliva, at different volume ratios, namely, 75:25, 50:50, and 25:75%, v/v.

Residual monomers released from dental materials were quantified after certain time intervals. Aliquots of 20 μL were taken for HPLC analysis after 24 hr, 7 d and 14 d. Respective chromatograms are presented in Figure 3A and 3B. Identification of released monomers was performed by the use of a photodiode array detector in a separate LC system, in addition to the retention time criterion.

Concentrations of monomers released from dental materials after the immersion of specimens in different time intervals in mixtures at different volume ratios are shown in Figure 4A-D comparatively.

From the results illustrated in Figure 4, it can be concluded that the amount of BIS-GMA eluted from the specimens increased with increasing immersion time of specimens in ethanol:water and ethanol:artificial saliva solution with volume ratio 50:50%, v/v. The monomer BIS-GMA was released in detectable quantities in organic solvents rather than in aqueous media.

As shown in Figure 4, BIS-GMA shows a decrease in concentration in artificial saliva solution 25:75 (v/v) at 14 d compared to results at 7 d. BIS-GMA in water 50:50 (v/v) shows significant release at 7 and 14 d, but the trend is not observed in the 25:75 (v/v) or 75:25 (v/v) solutions. The decrease in concentration of BIS-GMA in artificial saliva solution after 14 d of immersion is attributed to the fact that this solution contains corrosion

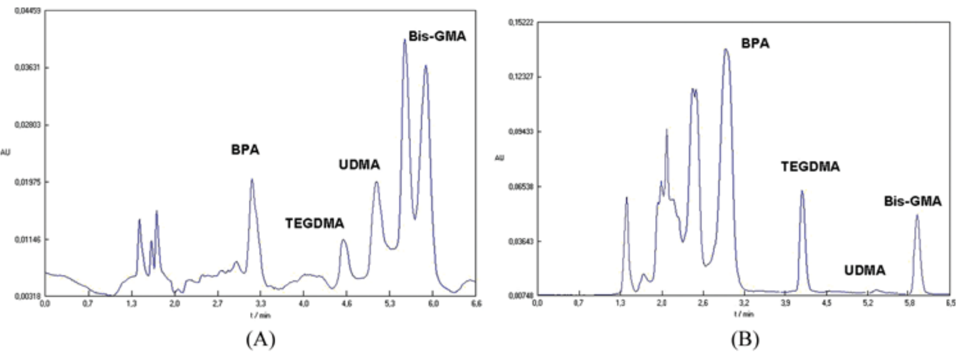

FIGURE 3 HPLC chromatogram of released compounds after 14 days of discs immersion in: A) ethanol:water 50:50%, v/v; and B) ethanol:saliva 50:50%, v/v. (Color figure available online.)

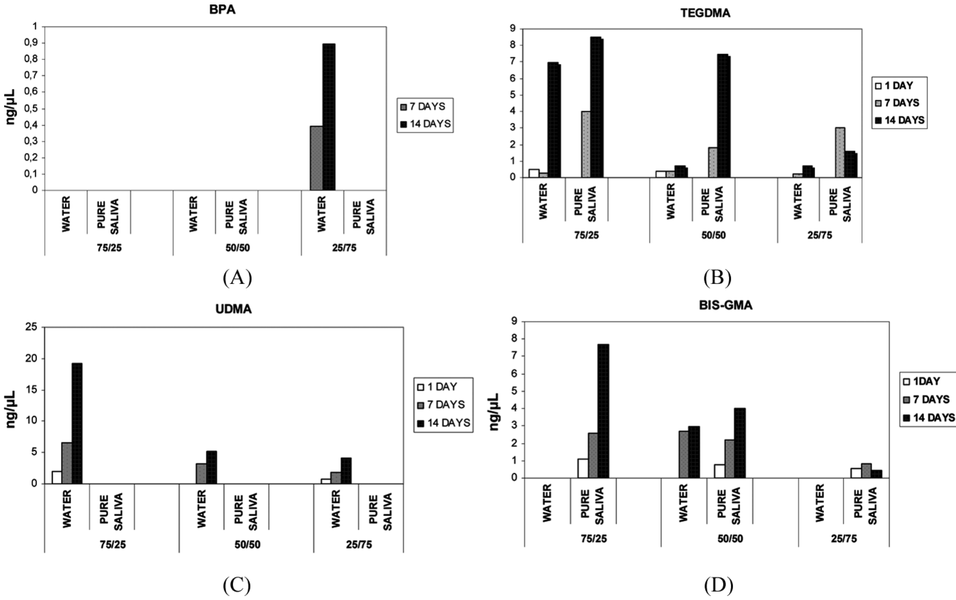

FIGURE 4 Concentration of monomers released after immersion of specimens in different solutions. A) BPA, B) TEGDMA, C) UDMA, and D) BIS-GMA.

inhibiting factors that act by forming an inhibiting layer on the surface of the material.[24]

On the other hand, TEGDMA amounts eluted from the specimens do not exhibit a significant difference in the various immersion solutions (ethanol:water). The only immersion solution that eluted a significant amount of this monomer is the solution with a volume ratio 75:25%, v/v. Although this molecule is hydrophobic, it was released in water mixtures in detectable quantities.

The monomer UDMA was released at higher concentrations with increased time of immersion. Although this compound is also hydrophobic, it was released in detectable quantities in aqueous media. Generally, the monomer UDMA released in greater quantities in organic solvents.

Finally, release of BPA was more profound in more aqueous mixtures. Similar results were obtained using ethanol-artificial saliva mixtures.

The hydrophobic monomer BIS-GMA was eluted in greater quantities in a solution with a volume ratio 75:25%, v/v, and the concentrations increased with the immersion time. Also, detectable amounts eluted in ethanol: artificial saliva solution with volume ratio 50:50%,v/v.

BPA, which is a degradation product of the BIS-GMA, could not be detected in a solution of ethanol:artificial saliva as it was released in very small quantities. Nevertheless, BPA is expected to exist as a monomer from the elution process since BIS-GMA has already been detected.

TEGDMA was released at higher rates in solution with volume ratio 75:25%, v/v, but also gave detectable quantities in solutions with volume ratio 50:50%, v/v and 100% pure artificial saliva. After the first 7 d of specimens immersion in ethanol:artificial saliva solution (75:25%, v/v), the concentration of TEGDMA that was released was higher than that of BIS-GMA.

Generally, the rate and the extent of elution appear to be greater in an organic solvent, such as ethanol:water (75:25%, v/v), as compared with elution into 100% water or 100% artificial saliva. This difference can be attributed to the greater ability of the organic solvent to penetrate and swell the polymeric network, facilitating the release of unreacted and leachable components. As the solvent penetrates the matrix and expands the openings between chains, monomers diffuse out.

Finally the low molecular weight monomers (TEGDMA) have more capacity mobility compared with higher molecular weight monomers (UDMA, BIS-GMA) and thus elute faster. Additionally, the results show that organic solvents are more aggressive in introducing the release of monomers from dental polymeric materials.

CONCLUSIONS

A simple and rapid method for the simultaneous determination of BPA, TEGDMA, UDMA, and BIS-GMA from dental polymeric materials in ethanol and artificial saliva solutions is described herein within 6 min. The developed method was validated in terms of selectivity, linearity, accuracy, precision, and sensitivity. Repeatability and between-day precision revealed RSD values lower than 11.2%. The relative errors ranged from −13.8% to 3.9%. In saliva the LOQ was 1.2–3.6 ng/μL.

The applicability of the method was confirmed by studying the residual monomers that can be released from dental polymeric materials after exposure in different solvent mixtures. The rate and extent of elution appear to be greater in an organic solvent, such as ethanol:water (75:25%, v/v), as compared with elution into 100% water, or 100% artificial saliva. This difference can be attributed to the greater ability of the organic solvent to penetrate and swell the polymer network, facilitating the release of unreacted and leachable components. As the solvent penetrates the matrix and expands the openings between polymer chains, monomers diffuse out.

To the best of our knowledge this is the first HPLC method for the simultaneous determination of BPA, TEGDMA, UDMA, and BIS-GMA from dental materials. No sophisticated equipment is necessary and a universal detection technique commonly used in every laboratory is sufficient to provide information for toxicological problems or allergies. For all presented

benefits this method is suitable and efficient for the simultaneous determination of unreacted monomers from dental polymeric materials.

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