Simultaneous analysis of bisphenol A based compounds and other monomers leaching from resin-based dental materials by UHPLC–MS/MS

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Abbreviations

2,2-Bis-(4-(3 methacryloxypropoxy) phenyl) propane (BisPMA)

2-hydroxyethyl methacrylate (HEMA)

2-Propenoic acid, (octahydro-4,7-methano-1H-indene-5-diyl)bis(methyleneiminocarbonyloxy-2,1-ethanediyl) ester (TCD-DI-HEA)

Bisphenol A (BPA)

Bisphenol A diglycidyl methacrylate (BisGMA)

Camphorquinone (CQ)

Ethoxylated bisphenol A glycol dimethacrylate (BisEMA)

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Higher limit of quantification (HLOQ)
Lower limit of quantification (LLOQ)
Triethylene glycol dimethacrylate (TEGDMA)
Urethane dimethacrylate (UDMA)

Keywords

dental materials, liquid chromatography, mass spectrometry, monomers, saliva

Abstract

Resin-based dental materials have raised debates concerning their safety and biocompatibility, resulting in a growing necessity of profound knowledge on the quantity of released compounds into the oral cavity. In this context, the aim of this study was to develop a comprehensive and reliable procedure based on liquid chromatography with mass spectrometry for the simultaneous analysis of various leached compounds (including bisphenol A based compounds) in samples from in vitro experiments. Different experiments were performed to determine the optimal analytical parameters, comprising mass spectrometry parameters, chromatographic separation conditions and sample preparation. Four internal standards were used: deuterated diethyl phthalate and bisphenol A (commercially available), and deuterated analogues of triethylene glycol dimethacrylate and urethane dimethacrylate (custom-made). The optimized method was validated for linearity of the calibration curves and the associated correlation coefficient, lower limit of quantification, higher limit of quantification, and intra- and inter-assay accuracy and precision. Additionally, the developed liquid chromatography with tandem mass spectrometry method was applied to the analysis of leaching compounds from four resin-based dental materials. The results indicated that this method is suitable for the analysis of different target compounds leaching from dental materials. This method might serve as a valuable basis for quick and accurate quantification of leached compounds from resin-based dental materials in biological samples.

1 Introduction

For over 150 years, amalgam has been used as the standard filling material, delivering a good clinical performance. However, its use has decreased over the past decades due to concerns about environmental mercury pollution [1] and unsatisfactory esthetics [2, 3]. As a result, the World Health Organization (WHO) advises to phase-down the use of dental amalgam [4] and to use alternative filling materials, such as resin-based dental composites (so-called ‘white fillings’), to restore the function and morphology of missing tooth structures. The use of light-curing resin composites in combination with resin-based adhesive systems led to a ‘revolution’ in restorative dentistry, mainly because of esthetic reasons [5]. Dental materials typically consist of an organic resin-based matrix, comprising various methacrylate monomers, additives (e.g. photo-initiators and others) and inorganic filler particles, constituted mainly of amorphous and colloidal silica or glasses [6].

With the increasing use of resin-based dental materials, several concerns regarding the safety and biocompatibility have been raised [6]. For instance, in clinical circumstances incomplete polymerization (either chemically or light initiated) may occur. The degree of conversion may vary between 50 and 70% for composites and between 65 and 90% for adhesive systems [7, 8]. As a result of the incomplete polymerization, together with intra-oral biodegradation processes, monomers, additives and filler components can leach out from the resin-based dental materials into

the oral environment. The release of compounds from resin-based dental materials might lead to adverse effects in local tissues (oral mucosa, gingival, pulp) as well as systemic effects [9]. Based mainly on in vitro studies, these adverse effects can be divided in either allergic [10] or toxic responses (cytotoxicity [11–17], genotoxicity and mutagenicity [17–25], and even reproductive toxicity [26, 27]). To date, the most commonly reported clinical adverse effects are allergic reactions, skin problems, oral ulcers, burning sensation, pain and post-operative sensitivity [10, 28]. Given the constant increase in the use of resin-based materials, a large population may be affected. Hence, attention should be paid to the relative biocompatibility of these dental restorative materials. There is a growing necessity to bridge the gap between the current alarming literature and the limited clinical observations. This can only be achieved by gaining knowledge on the quantity of compounds leaching from resin-based dental materials into the oral cavity [29].

A first step in the evaluation of dental materials biocompatibility is the assessment of the amount of potential leaching compounds in oral cavity, based on leaching experiment in a controlled environment. Thus, standardized blocks of resin-based materials are immersed in an extraction solvent for a specified time period and the extract is analyzed for various leached compounds. Extraction solvents typically used are: (1) water or aqueous mixtures, such as cell culture media, saliva or buffer solutions, to simulate clinical relevant conditions; and (2) organic solvents, usually ethanol- or methanol-based, to simulate worst-case scenarios [6]. Nowadays, the elution of the different compounds from dental materials tend to be analyzed more and more by liquid chromatographic methods, such as HPLC [30–34] and LC-MS/MS [35, 36]. Nevertheless, researchers in the dentistry field tend to stay vague about details on the used analytical parameters and often proper method validation is lacking. Nowadays, hyphenated techniques are required to target multiple analytes and to provide reliable results within a short time period with low quantification limits. The use of ultra UHPLC in combination with a tandem MS detector appears to be adequate to fulfill these requirements, in terms of speed, sensitivity and selectivity [37].

In this context, the aim of the present study was to develop a comprehensive and reliable procedure which allows the simultaneous analysis of the bisphenol A (BPA)-based compounds and other leaching contemporary monomers in three frequently used extraction solvents: water, artificial saliva and ethanol. Different experiments were performed to determine the optimal analytical parameters, comprising MS parameters, chromatographic separation conditions and sample preparation. Furthermore, the optimized analytical method was validated and the method suitability was evaluated in a controlled environment leaching experiment.

2 Materials and methods

2.1 Chemicals and materials

Alpha amylase, bisphenol A-d16, Celite 545, deuterium, diethyl phthalate-3,4,5,6-d4, acetic acid (LC-MS grade), formic acid (LC-MS grade), ammonium acetate (LC-MS ultra), ammonium formate (LC-MS), methanol (LC-MS grade), mucin from porcine stomach, potassium thiocyanate, sodium phosphate monobasic dehydrate and uric acid were purchased from Sigma-Aldrich (Diegem, Belgium). LC-MS-grade water was obtained from Biosolve (Valkenswaard, The Netherlands). Ethanol absolute and sodium hydroxide were purchased from Fisher Scientific (Aalst, Belgium). Potassium chloride and sodium chloride were obtained from VWR (Haasrode, Belgium). Urea was purchased from GE Healthcare Europe GmbH (Diegem, Belgium). Palladium on activated charcoal was obtained

from Acros Organics (Geel, Belgium). Clearfil SE Bond was obtained from Kuraray Noritake Dental Inc (Okayama, Japan). Tetric EvoFlow was obtained from Ivoclar Vivadent (Schaan, Liechtenstein).

2.2 Target compounds

Ethoxylated bisphenol A glycol dimethacrylate (BisEMA-3, BisEMA-6 and BisEMA-10), bisphenol A diglycidyl methacrylate (BisGMA), triethylene glycol dimethacrylate (TEGDMA) and urethane dimethacrylate (UDMA) were kindly provided by Esstech (Essington, PA, USA). 2,2-Bis-[4-(3 methacryloxypropoxy)phenyl]propane (BisPMA) was kindly provided by 3M ESPE (Seefeld, Germany). 2-Propenoic acid, (octahydro-4,7-methano-1H-indene-5-diyl)bis(methyleneiminocarbonyloxy-2,1-ethanediyl) ester (TCD-DI-HEA) was kindly provided by Dentsply (Konstanz, Germany). BPA, camphorquinone (CQ) and 2-hydroxyethyl methacrylate (HEMA) were purchased from Sigma-Aldrich (Diegem, Belgium). Characteristics of the target compounds are summarized in Table 1.

2.3 Custom-made internal standards and calibration standards

Four internal standards (IS) were used to overcome any potential sample manipulation losses: diethyl phthalate-3,4,5,6-d₄ (d₄-DEP) and bisphenol A-d₁₆ (d₁₆-BPA) were commercially available, while deuterated analogues of TEGDMA (IS-dT) and UDMA (IS-dU) were tailor-made according to the protocol described by Michelsen et al. [38]. Briefly, commercially available TEGDMA and UDMA were dissolved in methanol and reduced by hydrogenation with deuterium gas on 10% palladium on activated charcoal at atmospheric pressure over night. The product was filtered using Celite 545. The solvent was removed using a rotavapor (BÜCHI Labortechnik AG, Flawil, Switzerland).

For all target compounds, individual standard stock solutions with a concentration of 1000 µg/mL were prepared in absolute ethanol. For the IS, individual stock solutions of 500 µg/mL were prepared in absolute ethanol. Calibration standards were prepared in an ethanol/water mixture (1:1, v/v) with compound concentrations ranging from 0.5 to 5000 ng/mL for BisEMA-3, BisEMA-6, BisEMA-10, BisGMA, BisPMA, BPA, CQ, TCD-DI-HEA, TEGDMA, UDMA; and from 100 to 10000 ng/mL for HEMA. The concentration of the four IS was 1000 ng/mL. The calibration curves consisted of thirteen calibration points to ensure coverage of the full studies concentration range.

2.4 Apparatus and operating conditions

A Waters® Micromass Quattro Premier Mass Spectrometer (Waters, Milford, MA, USA) equipped with ESI was used for all sample analysis. The source temperature was set at 80°C and the desolvation temperature was set at 400°C. Argon was used as the collision gas (50 L/h) and nitrogen as the desolvation gas (800 L/h). Nitrogen was also used as nebulization gas. Multiple reaction monitoring (MRM) parameters for the analysis of the target compounds and the IS are summarized in Table 2. For each compound, the MRM transition with the highest measured response was set as the 'quantifier transition'. Other transitions were used as 'qualifier transitions' for confirmation. Note that the analysis was performed in positive ionization mode except for BPA and d₁₆-BPA.

Standard mixture or samples (10 µL) were injected on to an Acquity UHPLC BEH C₁₈ column (50 mm x 2.1 mm, 1.7 µm) (Waters, Milford, MA, USA), kept at a temperature of 40°C. An optimal chromatographic separation was achieved using a mixture of 2 mM ammonium acetate buffer, pH 5.6 (A) and methanol (B), upon the following gradient: 0–0.2 min, 15% B; 0.2–0.4 min, 15–60% B;

0.4–0.6 min, 60% B; 0.6–1.4 min, 60–95% B; 1.4–2.5 min, 95% B; 2.5–3.0 min, 95–15% B; 3.0–3.5 min, 15% B. The flow rate was set at 0.35 mL/min.

2.5 Sample preparation

An aliquot of 200 µL of the samples, from the monomer leaching experiment (see below), was taken and diluted to reach a mixture of ethanol/water or ethanol/artificial saliva (1:1, v/v). The artificial saliva samples were further acidified with 0.1% formic acid. Finally, 10 µL aliquots of the samples were injected into the UHPLC–MS/MS system. The samples with concentrations exceeding the calibration range were further diluted.

2.6 Method performance and validation

The developed method was validated for linearity of the calibration curves and the associated correlation coefficient R^2 , lower limit of quantification (LLOQ), higher limit of quantification (HLOQ), and intra- and inter-assay accuracy and precision in accordance with international rules [39–41].

2.6.1 Linearity and limits of quantification

The calibration standards were freshly prepared and analyzed the same day. The calibration curves were fitted by linear regression analysis on the peak area ratios of the target compounds to the IS, against the different nominal concentrations of the target compounds. The fitting of the calibration curves was evaluated using the error on the calculated concentrations expressed as percentage of target concentrations (% of target) and the correlation coefficient R^2 of the calibration curves. According to the abovementioned international rules, the deviation of the measured concentration from the nominal concentrations should not exceed 20%.

2.6.2 Intra-assay and inter-assay accuracy and precision

To ensure correct quantification, intra-assay accuracy and precision were evaluated by determining the compound concentrations in five replicates at low, medium and high concentration levels with the lowest level being the LLOQ and the highest level being the HLOQ. The intra-assay accuracy was calculated as the error to the nominal concentrations (% of target). The intra-assay precision was calculated as the RSD. Inter-assay accuracy and precision were assessed by analyzing the calibration standards in three separate days. The precision determined at each concentration should not exceed 20% while the accuracy should be within 80–120%.

2.6.3 Selectivity

All target compounds were analyzed using compound-specific MRM, leading to a reduction of interferences from other compounds in the sample. Moreover, besides a quantification transition ('quantifier'), at least one additional transition ('qualifier') was used for the confirmation of each of the target compounds. Additionally, correct identification of target compounds was ensured by checking the target specific retention times.

2.7 Monomer leaching experiment

The suitability of the developed method was evaluated by analyzing the eluted compounds from four different contemporary dental materials: two resin-based composites (Filtek Supreme XTE and Venus Pearl from 3M ESPE, Seefeld, Germany and Heraeus Kulzer, Hanau, Germany, respectively)

and two adhesive systems (Clearfil SE and OptiBond FL from Kuraray Medical, Tokyo, Japan and Kerr, Orange, CA, USA, respectively). The compositions of these dental materials were provided by the manufacturer. Filtek Supreme XTE universal nanocomposite containing BisGMA, UDMA, TEGDMA and BisEMA-6 resins, while Venus Pearl is a nano-hybrid composite which is predominantly based on TCD-DI-HEA and UDMA. Clearfil SE Bond is self-etching bonding agent of which the bond contains HEMA, BisGMA, CQ, among other components. OptiBond FL is an adhesive bonding system with a gold-standard reputation after more than a decade on the market and contains HEMA and BisGMA. Specimen disks (6 mm diameter and 2 mm thickness for composites; 5 mm diameter and 0.5 mm thickness for adhesive systems) were prepared in custom-made Teflon molds. For the composites, the molds were sandwiched between two glass slides with a thickness of 1 mm to reduce the oxygen inhibition layer, to ensure smooth surfaces and to avoid excess of material. For the adhesive systems, Teflon molds with a bottom (0.2 mm) were used. The materials were light-polymerized using a BluePhase curing unit (Ivoclar Vivadent, Schaan, Liechtenstein) (with an intensity of 880 mW/cm² and maximum wavelength of 470 nm) for 20 and 10 s for composites and adhesive systems, respectively. Immediately after polymerization, the specimen disks ($n = 3$ for each group) were immersed in 2 mL (composites) and 0.5 mL (adhesive systems) ethanol, water and artificial saliva for 24 h at 37°C. The artificial saliva was prepared according to the protocol described by Denys et al. [42]. Briefly, 1 L artificial saliva contains 896 mg potassium chloride (KCl), 888 mg sodium phosphate monobasic dehydrate (NaH_2PO_4), 200 mg potassium thiocyanate (KSCN), 298 mg sodium chloride (NaCl), 1.8 mL sodium hydroxide (NaOH) (1M), 200 mg urea, 15 mg uric acid, 50 mg mucin and 145 mg α -amylase. To avoid contamination, care was taken to use only glass pipettes and glass containers.

3 Results and discussion

3.1 Selection of target compounds

As mentioned above, dental materials typically consist of an organic resin-based matrix, consisting of several methacrylate monomers and additives (e.g. photo-initiators and others). Selection of the key target compounds was based on the general composition of resin-based dental materials, the constantly high prevalence of certain monomers, their specific function within the material and their potential adverse health effects, as briefly described hereafter.

CQ is the most commonly used photo-initiator and ensures the initiation of the polymerization reaction [43]. Most used monomers in the resin-based matrix, and probably the most studied, are the cross-linking dimethacrylates, such as TEGDMA, UDMA, BisGMA and BisEMA [44]. Occasionally, it is claimed that BPA can be found in dental composites, present as an impurity from the synthesis process and/or possibly as a degradation product of BPA-based monomers [27, 44]. Besides these well-known monomers, there are other more newly synthesized monomers, such as BisPMA and TCD-DI-HEA. The composition of dental adhesive systems is similar to resin-based composites. However, some specific monomers such as HEMA are included, because of their physicochemical properties.

Additionally, almost all the targeted monomers and additives have been shown to be cytotoxic *in vitro*, even if only in high concentrations [13–15]. Even more disturbing is the fact that sub-cytotoxic concentrations of frequently used monomers have been shown to seriously disrupt vital cell functions such as responses of the innate immune system, differentiation or induction of cell death through apoptosis [17, 45]. Moreover, endocrine disruptors came into the spotlights ever since it

was shown that BPA and BPA-derivatives can be detected in saliva from patients treated with resin-based dental sealants and that these compounds are able to cause estrogenic activity *in vitro* [46]. Endocrine disrupting chemicals or xenoestrogens interfere with endocrine functions by altering the physiological hormone levels and mimicking or blocking endogenous hormones. As a consequence, they may cause adverse developmental, reproductive, neurological, cardiovascular, immune and metabolic effects [47].

3.2 Optimization of the UHPLC–MS/MS operating conditions

The UHPLC–MS/MS method was optimized by: (i) selecting optimal mass spectrometric conditions, (ii) selecting optimal chromatographic conditions, (iii) developing an auspicious sample preparation. Since the probability of finding two compounds with the same transitions and the same retention time is virtually non-existent, special efforts were made to ensure efficient LC separation. Moreover, accurate selection of the mobile phase composition can be relevant to enhance the detector response [48].

3.2.1 Mass spectrometric conditions

Optimization of the MS parameters was achieved by direct infusion of individual standard solutions of the target compounds into the ESI source at a flow rate of 20 μ L/min. General optimized parameters included source temperature, desolvation temperature and gas flows. For each target compound, ionization voltages were further fine-tuned, creating the optimal conditions for ionization. The most sensitive MRM transition was typically selected for quantification ('quantifier') whereas at least one additional transition ('qualifier') was selected to provide a highly reliable confirmative method. A detailed description of the optimal MS/MS conditions is presented in Table 2.

3.2.2 Chromatographic conditions

Chromatographic conditions were adjusted to reach optimal resolution, peak shape and enhanced response. Variation of solvents flow rate (i.e. from 300 to 500 μ L/min) as well as of the separation gradient had a minimum influence on the chromatographic separation in comparison to the buffer type and pH value. Thus, the evaluation of the impact of different types of buffers (i.e. ammonium formate and ammonium acetate) and of their pH on the chromatographic performances was further studied (Fig. 1).

Buffers (i.e. ammonium formate and ammonium acetate buffers) classically used to adapt the pH of the mobile phase [49] were evaluated to reach low LOQs. Ammonium formate buffers, 10 mM (pH 4.7 or 6.2), resulted in poor chromatographic parameters as for both peak area and shape of the majority of the target compounds, except for BisEMA-3 and BisPMA. Unlike ammonium formate buffers, the use of ammonium acetate buffer 2 mM resulted in enhanced signals. Different pH values were further tested to reach the optimal conditions. A pH of 3.7 resulted in rather deprived outcomes, especially for CQ but also for TEGDMA, TCD-DI-HEA, BisGMA and the three BisEMA types. Between pH 7.8 and pH 5.6, only minor differences in peak area were observed. However, the peak intensity of both BPA and CQ were higher at pH 5.6. Thus, the final optimal chromatographic conditions were achieved using a mixture of ammonium acetate buffer, 2 mM (pH 5.6) and methanol.

3.3 Optimization of the sample preparation

During the method development, the sample treatment was also investigated. Our purpose was to avoid the variations caused by the different solvents used in elution experiments [30, 34, 35, 49, 50] or the potential necessity to prepare different standard calibration curves for each tested solvent individually. Two different approaches were tested: sample evaporation and sample dilution with a complementary solvent.

Preliminary testing demonstrated the effect of the solvent on the analytical signal. Standard mixtures containing different ratios of ethanol/water (ranging from 10–100% of ethanol) resulted in considerable changes of the response (peak area) (data not shown). Thus, the possibility to add to the individual samples a complementary solvent (i.e. water or ethanol) became obvious and was further evaluated. By treating the samples to reach a mixture of ethanol/water (1:1, v/v), enhanced analytical signals were obtained. However, for some target compounds (i.e. BPA, CQ, TEGDMA, UDMA and TCD-DI-HEA) further testing revealed dissimilarities between ethanol/water (1:1, v/v) and ethanol/artificial saliva (1:1, v/v) mixtures. For instance, the peak areas were diminished by a factor ranging from 2 to 10 (from TCD-DI-HEA to BPA) when replacing ethanol/water (1:1, v/v) with ethanol/artificial saliva (1:1, v/v). To overcome this problem 0.1% formic acid was added to the ethanol/artificial saliva (1:1, v/v) samples to have a similar pH value as for the mobile phase, leading to a signal comparable to the one obtained with the ethanol/water (1:1, v/v) samples (Fig. 2). Meanwhile, the other target compounds seemed not to be affected by the pH change in the sample. Beforehand, sample evaporation was tested using three individual solvents (ethanol, water and artificial saliva) containing the different target compounds at a concentration of approximately 5 µg/mL. Evaporation under nitrogen flow and subsequent re-suspension in the mobile phase resulted in substantial differences in compounds recovery. For instance, mean recovery values ranged from 6.5% (CQ) to 154.8% (BPA) for ethanol, from 0.7% (CQ) to 141.6% (BPA) for water and from 19.1% (CQ) to 132.9% (BPA) for artificial saliva. Only a few compounds, such as TEGDMA and UDMA, provided results with recovery values around 70–90%. As a consequence, sample evaporation before analysis was not further considered.

3.4 Multi-residue method validation

3.4.1 Linearity and limits of quantification

Calibration curves of the individual target compounds were obtained using stable-isotope-labeled IS and were linear within the domain of calibration (Table 3). The lower and higher levels of the domain of calibration were represented by the LLOQ and the HLOQ, respectively. For all compounds, the correlation coefficients R^2 of the regression equations exceeded the value 0.99, with the exception of TEGDMA (0.9888) and CQ (0.9815). All tested concentrations within the domain of calibration, that is between the LLOQ and the HLOQ, were showing appropriate values for both accuracy (within the interval 80–120%) and precision (RSD lower than 20%). These results indicated a good correlation between the measured response (peak area) and the nominal concentration of the target compounds.

In human saliva, it is expected that the concentration of the eluates from resin-based dental composite will be low due to continuous saliva flow. Unfortunately, only a few studies on the *in-vivo* quantification of compounds leached from resin-based dental materials are available. Kingman et al. [52] reported that their LC–MS method for the analysis of 6 different BPA-related compounds in saliva had LLOQs as low as 0.9, 1.2 and 2.7 ng/mL for BPA, TEGDMA and BisGMA, respectively. The

study of Michelsen et al. [53] was able to detect different monomers in saliva samples. The lowest concentrations were 15, 28 and 4 ng/mL for HEMA, BisGMA and UDMA, respectively. These results point towards low concentrations of leached compounds in saliva of patients. Thus, our aim was to reach LLOQs as low as possible and consequently sufficient sensitivity. In this sense, for BisEMA, BisGMA, BPA, CQ, HEMA, TEGDMA and UDMA, the LLOQs reached using our method are lower or similar to the ones reported in literature [31–36, 53–55]. To the best of our knowledge, no data on the LLOQs for the other target compounds was available for comparison. Despite the fact that these compounds are nonetheless analyzed by different techniques, there is a lack of precise information on the LLOQ since some authors fail to mention details on the used analytical method.

3.4.2 Intra- and inter-assay accuracy and precision

Intra-assay accuracy and precision were evaluated by analyzing three different concentration levels in five replicates. The intra-assay accuracy (expressed as percentage of the target concentrations) varied between 93 and 118%, except for HEMA (level 2 with a value of 126%), while the precision (expressed as RSD) varied between 2 and 18% with the highest values found for BisGMA and CQ. The inter-assay accuracy and precision were validated by analyzing the same calibration standards at three separate batches. Inter-assay accuracy varied between 86 and 124%, and intra-assay precision did not exceed 17%, with the exception of TCD-DI-HEA at level 1, where the precision was 22%. These results indicate that the developed method is highly reproducible and accurate.

3.5 Monomers leaching experiment

Upon method optimization and validation, the suitability of the developed UHPLC-MS/MS method was further evaluated by analyzing the leached compounds from four conventional resin-based dental materials (i.e. Filtek Supreme XTE, Venus Pearl, Clearfil SE Bond and OptiBond FL) submitted to an in vitro elution experiment using 1 mL ethanol, water and artificial saliva as extraction media. Absolute ethanol as extraction solvent should be considered as the worst-case scenario. Ethanol and ethanol/water mixtures are able to penetrate the methacrylate polymer network, resulting in more sorption and swelling [57]. Despite the fact that ethanol as an extraction solvent does not resemble the clinical situation, it might allow the assessment of all possible leachable substances. Unlike for ethanol, the majority of the tested eluates are released in lower amount in water-based solutions [6]. Water and artificial saliva were incorporated in this study to simulate a clinically relevant situation for testing of resin-based dental materials.

The results of the in vitro elution experiment are shown in Table 4. As expected, immersion of the material disks in pure ethanol induced a higher release of compounds compared to the other extraction solutions. No significant difference was observed between water and artificial saliva. Fig. 3 presents the obtained UHPLC-MS/MS chromatograms of the compounds eluted from the four different resin-based materials when using artificial saliva as extraction solvent.

All tested resin-based materials released CQ and BisGMA, while only the adhesive systems released HEMA. According to the data provided by the manufacturer, Filtek Supreme XTE contains BisGMA, UDMA, TEGDMA and BisEMA-6 resins. This *in-vitro* elution experiment could demonstrate the presence of these four monomers, together with CQ and the different forms of BisEMA (i.e. BisEMA-3 and BisEMA-10). These results are in accordance with previous studies [58–62]. Venus Pearl is based on TCD-DI-HEA and UDMA. Nonetheless, besides TCD-DI-HEA and UDMA, also CQ, TEGDMA, and small amounts of BisGMA were found in the extraction solutions. To the best of our knowledge,

no elution studies were performed on the composite Venus Pearl. However, monomer elution from Venus Diamond, a composite with comparable composition, was studied. Analysis with GC-MS revealed the presence of TEGDMA and CQ [61], while more targeted analysis with a HPLC technique was able to detect TEGDMA (58.38 µg/mL), UDMA (92.40 µg/mL), BisEMA (768.41 µg/mL), but failed to detect BisGMA when 1.5 mL ethanol/water (70:30, v/v) was used as extraction solution [62]. Clearfil SE Bond contains HEMA, BisGMA, CQ, among other components. The results showed the presence of HEMA, CQ, BisGMA, but also of TEGDMA and UDMA. OptiBond FL comprises HEMA and BisGMA. CQ and even small amounts of BisEMA were found when pure ethanol was used as the extraction solvent. Adhesive systems are less common subjected to elution studies. Studies on monomer elution on Clearfil SE revealed the presence of HEMA and BisGMA [63], while results for OptiBond FL demonstrated leaching of HEMA in both 75% ethanol and human saliva [63, 64]. Nonetheless, it is challenging to compare results from different elution studies due to the heterogeneity of the elution protocols used (e.g. different size of dental material samples, type of extraction solution, volume of extraction solution, and analytical procedures).

4 Concluding remarks

In this study, an UHPLC-MS/MS method for the simultaneous analysis of 11 leaching compounds from resin-based dental materials was developed and successfully validated. The method included a simple sample preparation step that allowed the analysis of various leaching compounds in three frequently used extraction solvents: ethanol, water and artificial saliva. Furthermore, this method resulted in an adequate accuracy and precision for both inter- and intra-assay validation, with overall LLOQs lower or comparable to previous described analytical methods targeting a limited number of compounds. Additionally, the developed method was applied to the analysis of eluted compounds from four conventional dental materials. The obtained results indicated that the developed method is suitable for the analysis of the different target compounds leaching from resin-based dental materials. This method might serve as a valuable basis for simple, quick and accurate quantification of leached compounds from resin-based dental materials in biological samples.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure captions

Fig. 1. Impact of different mobile phase solvents on the UHPLC–MS/MS performance.

Representative UHPLC–MS/MS chromatograms of a standard mixture analyzed using the following mobile phases in combination with methanol: (A) 2 mM ammonium acetate buffer pH 5.6, (B) 2 mM ammonium acetate buffer pH 7.8, (C) 2 mM ammonium acetate buffer pH 3.7, (D) 10 mM ammonium formate buffer pH 6.2, or (E) 10 mM ammonium formate buffer

pH 4.7. For better visualization different zoom factors, presented hereafter in parentheses, were used. In the chromatograms are presented the next analytes as follows: [1] CQ 5.065 $\mu\text{g/mL}$ (x200); [2] BPA 5.160 $\mu\text{g/mL}$ (x800); [3] TEGDMA 5.105 $\mu\text{g/mL}$; [4] TCD-DI-HEA 5.100 $\mu\text{g/mL}$ (x4); [5] UDMA 5.140 $\mu\text{g/mL}$; [6] BisGMA 5.260 $\mu\text{g/mL}$; [7] BisEMA-10 5.100 $\mu\text{g/mL}$; [8] BisEMA-6 5.065 $\mu\text{g/mL}$; [9] BisEMA-3 5.205 $\mu\text{g/mL}$; [10] BisPMA 5.125 $\mu\text{g/mL}$ (x1/2).

Fig. 2. The effect on the analytical signal when adding 0.1% formic acid (FA) to the sample solvent containing ethanol (EtOH) in combination with water (H₂O) or artificial saliva (AS). The relative response (expressed as percentage, relative to EtOH/H₂O (1;1, v/v) samples) for the different target compounds when analyzed in different sample solvents. The results are represented as mean values with the bars representing the standard error of the mean ($n=5$).

Fig. 3. Chromatograms of eluted target compounds in the artificial saliva samples obtained with the in vitro elution experiment. For better visualization different zoom factors, presented hereafter in parentheses, were used. (A) The calibration standard consisted of a mixture of ethanol/water (1:1, v/v) containing the following compounds: [1] HEMA 1051 µg/mL; [2] BPA 103.2 µg/mL (x100); [3] CQ 101.3 µg/mL (x10); [4] TEGDMA 102.1 µg/mL (x1/2); [5] TCD-DI-HEA 102.0 µg/mL; [6] UDMA 102.8 µg/mL; [7] BisGMA 105.2 µg/mL; [8] BisEMA-10 102.0 µg/mL; [9] BisEMA-6 101.3 µg/mL; [10] BisEMA-3 104.1 µg/mL; [11] BisPMA 102.5 µg/mL (x1/10). (B) Filtek Supreme XTE: CQ (10x), TEGDMA (x1/4), UDMA (x1/2), BisEMA-10, BisEMA-6 and BisEMA-3. (C) Venus Pearl: CQ (10x), TEGDMA (x1/16), TCD-DI-HEA (x1/4) and UDMA (x1/2). (D) OptiBond FL: HEMA, CQ, BisGMA. (E) Clearfil SE: HEMA, CQ, BisGMA (x1/2).

Table 1

Description of target compounds.

Compound	Structure	Molecular formula
CAS number		Molecular weight
HEMA		$C_6H_{10}O_3$
868-77-9		130.1
CQ		$C_{10}H_{14}O_2$
10373-78-1		166.2
BPA		$C_{15}H_{16}O_2$
80-05-7		228.3
TEGDMA		$C_{14}H_{22}O_6$
109-16-0		286.3
TCD-DI-HEA		$C_{24}H_{34}N_2O_8$
861437-11-8		504.6
UDMA		$C_{23}H_{38}N_2O_8$
72869-86-4		470.6
BisGMA		$C_{29}H_{36}O_8$
1565-94-2		512.6
BisEMA-10		$C_{43}H_{64}O_{14}$
41637-38-1		805.0 $n + m = 10$
BisEMA-6		$C_{35}H_{48}O_{10}$
41637-38-1		628.7 $n + m = 6$
BisEMA-3		$C_{29}H_{36}O_7$
41637-38-1		496.6 $n + m = 3$
BisPMA		$C_{29}H_{36}O_6$
27689-12-9		480.6

Table 2

MS/MS parameters for the analysis of the target compounds and the internal standards.

Compound	R _t (min)	Ionization mode	Quantifier			Qualifier		
			Transition	Cone (V)	Collision (V)	Transition	Cone (V)	Collision (V)
HEMA	0.89	ES+	130.8 → 130.8	18	4	130.8 → 69.0	18	19
CQ	1.13	ES+	167.0 → 83.0	22	21	167.0 → 139.0	22	12
<i>d16-BPA</i>	1.29	ES-	241.0 → 222.9	40	20			
BPA	1.31	ES-	226.9 → 211.8	40	20	226.9 → 132.8	40	25
<i>d4-DEP</i>	1.33	ES+	227.6 → 153.2	15	18			
TEGDMA	1.34	ES+	304.0 → 113.0	15	7	304.0 → 287.0	15	7
<i>IS-dT</i>	1.38	ES+	295.0 → 117.0	20	14			
TCD-DI-HEA	1.49	ES+	479.5 → 407.0	26	15	501.0 → 357.0	26	29
UDMA	1.56	ES+	493.7 → 493.7	50	10	488.0 → 471.0	9	10
<i>IS-dU</i>	1.60	ES+	503.0 → 251.0	42	38			
BisGMA	1.74	ES+	530.3 → 277.1	8	23	513.3 → 495.0	8	10
						535.3 → 535.3	50	15
BisEMA-10	1.84	ES+	778.5 → 335.0	25	30	822.5 → 335.0	25	30
BisEMA-6	1.88	ES+	602.5 → 291.0	20	15	646.5 → 291.0	20	20
BisEMA-3	1.89	ES+	470.2 → 247.0	30	15	514.4 → 247.0	30	18
						558.2 → 291.0	30	20
BisPMA	2.07	ES+	498.2 → 261.0	23	15	498.2 → 127.0	23	24

R_t: retention time; V: voltage.

Table 3

Intra-assay and inter-assay validation results.

Compound	Linearity (R ²)	Domain of calibration (ng/mL)	Intra-assay		Inter-assay	
			Accuracy (% of target)	Precision (RSD%)	Accuracy (% of target)	Precision (RSD%)

			(n=5)			(n=5)			(n=3)			(n=3)		
		LLOQ-HLOQ	Level			Level			Level			Level		
			1 ^a	2 ^b	3 ^c	1 ^a	2 ^b	3 ^c	1 ^a	2 ^b	3 ^c	1 ^a	2 ^b	3 ^c
HEMA	0.9910	200-5000	114	126	108	11	5	6	122	112	110	9	5	4
CQ	0.9815	50-500	118	101	109	18	17	7	86	94	99	16	9	5
BPA	0.9944	50-500	93	102	94	13	11	9	97	102	93	11	10	17
TEGDMA	0.9888	5-100	116	96	105	4	4	3	108	95	104	10	5	4
TCD-DI-HEA	0.9956	5-200	118	104	107	5	8	2	124	103	106	22	9	3
UDMA	0.9947	5-100	96	106	97	11	4	10	96	101	100	10	7	8
BisGMA	0.9956	10-100	114	114	105	16	3	3	120	106	102	13	10	5
BisEMA-10	0.9969	5-100	102	93	101	6	2	4	105	94	103	5	8	7
BisEMA-6	0.9967	2-100	110	99	99	5	6	2	112	96	100	11	5	5
BisEMA-3	0.9966	2-100	108	101	100	6	4	2	112	105	104	8	4	3
BisPMA	0.9934	5-100	114	95	102	6	2	4	109	99	103	7	5	5

a – Level 1 (LLOQ) corresponds to the following concentrations: 2 ng/mL BisEMA-3; 2 ng/mL BisEMA-6; 5 ng/mL BisEMA-10; 10 ng/mL BisGMA; 5 ng/mL BisPMA; 50 ng/mL BPA; 50 ng/mL CQ; 200 ng/mL HEMA; 5 ng/mL TCD-DI-HEA; 5 ng/mL TEGDMA; 5 ng/mL UDMA.

b – Level 2 corresponds to the following concentrations: 10 ng/mL BisEMA-3; 10 ng/mL BisEMA-6; 20 ng/mL BisEMA-10; 50 ng/mL BisGMA; 10 ng/mL BisPMA; 200 ng/mL BPA; 200 ng/mL CQ; 1000 ng/mL HEMA; 20 ng/mL TCD-DI-HEA; 10 ng/mL TEGDMA; 20 ng/mL UDMA.

c – Level 3 (HLOQ) corresponds to the following concentrations: 100 ng/mL BisEMA-3; 100 ng/mL BisEMA-6; 100 ng/mL BisEMA-10; 100 ng/mL BisGMA; 100 ng/mL BisPMA; 500 ng/mL BPA; 500 ng/mL CQ; 5000 ng/mL HEMA; 200 ng/mL TCD-DI-HEA; 100 ng/mL TEGDMA; 100 ng/mL UDMA.

RSD: relative standard deviation

Table 4

Concentration of detected monomers eluted from dental materials in 3 different extraction

solvents.

		Concentration of eluted monomers (μM)								
Dental material	Extraction solvent	HEMA	CQ	TEGDMA	TCD-DI-HEA	UDMA	BisGMA	BisEMA-10	BisEMA-6	BisEMA-3
Resin-based composite										
Filtek Supreme XTE	EtOH	N.D.	8.37±0.95	3.27±0.01	N.D.	32.64±0.03	31.77±2.64	12.97±0.01	27.60±0.01	21.30±0.01
	H ₂ O	N.D.	7.19±0.29	2.95±1.75	N.D.	8.81±3.62	1.20±1.35	0.32±0.17	0.17±0.08	0.11±0.01
	AS	N.D.	8.43±0.17	2.66±2.31	N.D.	9.35±0.23	1.91±0.07	0.58±0.01	0.83±0.01	0.59±0.01
Venus Pearl	EtOH	N.D.	11.88±0.88	35.30±0.01	301.60±0.01	56.11±0.04	1,07±2.86	0.27±0.01	0.79±0.01	0.63±0.01
	H ₂ O	N.D.	6.81±0.29	9.81±1.76	51.51±0.51	7.00±3.82	0.16±1.43	N.D.	N.D.	N.D.
	AS	N.D.	10.08±0.90	7.30±6.41	43.73±32.08	5.69±4.08	0.07±1.61	N.D.	N.D.	N.D.
Adhesive system										
OptiBond FL	EtOH	792.68±31.56	7.96±0.35	N.D.	N.D.	N.D.	172.92±56.53	N.D.	0.05±0.01	N.D.
	H ₂ O	327.23±4.91	8.17±0.23	N.D.	N.D.	N.D.	5.74±0.60	N.D.	N.D.	N.D.
	AS	323.75±5.96	11.01±0.95	N.D.	N.D.	N.D.	4.82±0.49	N.D.	N.D.	N.D.
Clearfil SE BOND	EtOH	218.23±17.56	5.27±0.76	N.D.	N.D.	N.D.	67.35±8.79	0.24±0.00	0.66±0.01	0.51±0.01
	H ₂ O	173.54±8.75	9.18±0.11	N.D.	N.D.	N.D.	3.05±0.18	N.D.	N.D.	N.D.
	AS	N.D.	6.71±0.87	N.D.	N.D.	N.D.	2.54±1.54	N.D.	N.D.	N.D.

The results are represented as mean values $\pm$ SD (n=3).EtOH: ethanol; H₂O: water; AS: artificial saliva; N.D.: not detected (under the LOQ).