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ORIGINAL ARTICLE

HPLC analysis of potentially harmful substances released from dental filing materials available on the EU market

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Abstract

Introduction. Incomplete cross-linking of composite dental materials leads to their susceptibility to degradation in the environment of non-organic and organic solvents, contributing to the release of chemical compounds which are potentially harmful to living organisms.

Objective. The aim of the study was an evaluation in *in vitro* conditions of releasing of potentially toxic substances from six dental composite materials available in EU countries.

Materials and methods. The following compounds released from the samples stored in water were analyzed: bisphenol A (BPA), triethylene glycol-dimethacrylate (TEGDMA), urethane dimethacrylate (UDMA) and ethylene glycol dimethacrylate (EDGMA). Analysis of the substances was performed with the use of high performance liquid chromatography, after the following incubation periods: 1 hour, 24 hours, 7 days and 30 days.

Results. Among the analyzed substances, after 1 hour of incubation, the highest average concentration was found for TEGDMA – 2045 μg cm^{-3} (in Herculite XRV material), after 24 hours – for UDMA 4.402 μg cm^{-3} (in Gradia Direct Anterior material) and after 7 and 30 days for TEGDMA: 8.112 and 6.458 μg·cm^{-3} respectively (in Charisma material).

Conclusions. The examined composites used for reconstruction of hard tissues of teeth remain chemically unstable after polymerization, and release potentially harmful substances in conditions of the present study. The dynamics of the releasing of potentially harmful substances is correlated with the period of sample storage in water.

Key words

Dental composite, bisphenol A (BPA), high performance liquid chromatography (HPLC)

INTRODUCTION

In most cases, the matrix of commercially available dental composites is made of a mixture of various monomers, the most common of which are: bisphenol A-glycidyl methacrylate, urethane dimethacrylate, triethylene glycol dimethacrylate or ethylene glycol dimethacrylate [1].

One of the phenomena responsible for unfavourable features of organic polymer matrix is its incomplete cross-linking which takes effect during polymerization initiated by a chemical reaction, or activated by visible light. Data published in available literature indicate that only about 32–76% of monomer double bonds participate in the polymerization process of dental materials. To-date, no dental material has been produced whose matrix would convert in 100% to create a stable space lattice [2, 3].

Incomplete cross-linking of composite dental materials leads to their susceptibility to degradation [4] in the environment of non-organic and organic solvents, contributing to release into the external environment of many chemical compounds which are potentially harmful to living organisms. According to data quoted by Bakopoulou *et al.* [5], more than 30 chemical compounds released from composites into the external environment have been identified. These substances include mainly monomers, resin comonomers or oligomers, hydrolysis products of the above products, fillers, initiators, catalysts and stabilizers of polymerization reaction, and metal ions. Some researchers believe that eluting non-polymerized ingredients of materials into solutions terminates after a few days to a few weeks from commencement of polymerization. However, it is difficult to explicitly prove what percentage of substances identified in composite eluates are particles not bonded during polymerization, and what percentage are products of material degradation during hydrolytic cleavage [6].

Particles of materials for filling cavities in the hard tissues of teeth may be present also outside the oral cavity environment. It has been demonstrated that they are found in the spray formed in the patient's oral cavity during dental treatment. They can penetrate to the nasopharynx, eyes, and settle on the skin of both the patient and medical staff [7]. Constant exposure to potentially harmful chemicals released outside the oral cavity during preparation of fillings may – apart from biological factors [8] present in the aerosol formed during dental treatment – constitute a potential professional risk factor for dentists and dental assistants.

Chemical compounds released from dental composites used for the reconstruction of hard tissues of teeth may

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act topically in the oral environment, as well as constitute a potential threat for the whole organism. Methacrylate monomers released from fillings may have an adverse effect on tooth pulp cells, causing a temporary inflammatory process of tissue or necrosis; they may also have an unfavourable impact on oral epithelium, contributing to the development of lichenoid-type lesions on mucous membrane. Substances released from dental composites into the external environment may also cause local hypersensitivity reactions. The adverse effect of chemical compounds included in dental composites has been confirmed in numerous studies performed on cell cultures and laboratory animals [9].

BPA is a monomer which is widely used in industry for production of polycarbonates. Polycarbonate plastic materials are used in many branches of industry in the manufacture of, among other things, panes, contact lenses, food wrappers, including disposable and multiple-use bottles, and elements of dummies for baby feeding. It is assumed that bisphenol A is a biologically-active chemical compound demonstrating – depending on the dose and exposure time – cytotoxic, parahormonal and mutagenic action. The compound does not solely act directly, impairing bodily functions of cells and organs, but also through confirmed parahormonal activity it can imitate hormones from the estrogen group [10]. Analysis of the results of laboratory tests conducted on ovocytes of domesticated animals indicates an impact of subtoxic BPA on the maturing and division of zygotes, and on impairment of their development [11].

Results of the study by Hugo *et al.* [12] suggest an existing association between environmental exposure to bisphenol A and occurrence of obesity in humans. The quoted authors confirmed a positive correlation between BPA presence in serum, and an impairment of adipose tissue cell metabolism by, among other things, increasing their resistance to insulin action.

The studies by Midoro-Horiuti *et al.* [13] performed on animals, demonstrated an association of exposure to bisphenol A with impairment of interneuron connection formation in the central nervous system, and with inducing chronic pathologies of the respiratory system. The results of the study by Ishido *et al.* [14] indicate the ease of bisphenol A expansion in the central nervous system. Results of tests on laboratory animals suggest that BPA has an adverse effect on growing living organisms, even in relatively low doses. Therefore, the phenomenon of bisphenol A release from dental materials requires thorough studies with regard to the safety of their use.

Studies conducted on tissue cultures demonstrated that TEGDMA contributes to increased concentration of free radicals in cell structures, causing their damage [15]. The results of the studies by Eckhardt *et al.* [16] suggest that this chemical compound significantly impairs the function of immunocompetent cells, including lymphocytes and monocytes, modulating the organism's immune response. In laboratory conditions, TEGDMA induces apoptosis of cells, and in higher doses causes tissue necrosis [17].

In studies conducted on cell cultures, cytotoxic and genotoxic action of UDMA was also confirmed [18].

EDGMA is a monomer commonly used in the chemical industry to improve chemical and physical properties of polymers, among other things, in order to increase their resistance to temperature and aggressive chemicals. In dentistry, it constitutes a component of composite materials for fillings, orthodontic adhesive resins and acrylic denture plates. EDGMA is believed to have the characteristics of a strong allergen [19].

OBJECTIVE

The aim of the study was an assessment of release of potentially harmful substances from composite filling materials available on the EU market in *in vitro* conditions.

MATERIALS AND METHOD

The following six composites used for the reconstruction of hard tissues of teeth were assessed: Gradia Direct Anterior (GC Corp., Japan), Arkon (Arkona, Poland), Filtek Z550 (3M, USA), Herculite XRV (Kerr Italia, Italy), Tetric Evo Ceram (Ivoclar – Vivadent, Lichtenstein), Charisma (Haraeus Kulzer, Germany). The listed composites came from Polish distribution sources and approved for sale on the European market.

Identification of potentially harmful chemical compounds. The release of potentially harmful chemical compounds from the examined composites used for reconstruction of hard tissues of teeth was assessed in four observation periods, i.e. after 1 hour, after 24 hours, after 7 days and after 30 days of storing the samples in water. The evaluated composites were placed in the hollows of teflon matrixes 5 mm in diameter and 2 mm in depth. When the matrix was full, each sample was polymerized for 40 seconds with light of 1,100 mW/cm^2 intensity from a LED 55 Curing Light (TPC Advanced Technology, USA). After completion of polymerization, the samples were removed from the matrices with the use of glass spatulas washed with 70% ethanol and HPLC grade water (Sigma Aldrich, USA) and placed in separate glass containers sealed with a Parafilm membrane (Brand GmBH, Germany) for 24 hours. The above-method was used to prepare 20 samples of each assessed material, which were then randomly divided into four groups (five samples in each group), corresponding to individual time periods of the planned experiment.

After 24 hours, the studied samples were placed in test tubes filled with 10cm^3 of water (HPLC grade) with added 0.05cm^3 of Antibiotic Antimicotic preparation (Invitrogen, USA), containing amphotericin B, streptomycin and penicillin, in order to avoid any contamination of the solutions with microorganisms. The samples were then placed in a Classic C-24 incubator shaker (New Brunswick Scientific, USA) oscillating at 112 cycles per minute at 37 °C. Five samples of each assessed composite were incubated in the above conditions respectively for: 1 hour, 24 hours, 7 days and 30 days. After removal from the incubator shaker, the studied samples were also removed from test tubes, and the obtained eluates frozen at the temperature of -8 °C. The samples were thawed directly before performing a chromatographic analysis in a water bath at 37 °C.

The following chemical compounds were identified in the water solutions obtained by the method specified above: bisphenol A (BPA), triethylene glycol-dimethacrylate (TEGDMA), urethane dimethacrylate (UDMA) and ethylene glycol dimethacrylate (EDGMA.

Determination of the decomposition product content released from the tested samples was performed by the RP HPLC (reversed phase high performance liquid chromatograpy).

Equipment and chromatographic conditions. Quantitative analysis was carried out using Shimadzu LC-10AT gradient system (Shimadzu Manufacturing Inc., USA), equipped with UV-Vis SPD-10A detector, SCL-10-A system controller, and CTO-10AC column oven. The column used was RP-18, 5-nm particle size, 250/4-mm Supelcosil DB (Supelco, USA). The eluents were A: HPLC-grade water, and B: HPLC-grade acetonitrile obtained from Merck. The gradient breakpoints are presented in Table 1. Temperature – 25°C, injection volume – 20 µL, and the flow rate – 1 mL/min. Before injection, the samples were filtered through a 0.45-µm filter Chromclean (Merck, Germany). The wavelength of detection was 205 nm. All determinations were performed in triplicate. The procedure used was a modified Manojlovic et al. [20] method. The gradient method was chosen because it allows simultaneous determination of all substances identified in the tested eluates. As external standards, HPLC standards (Sigma-Aldrich, USA) were used, listed in Table 2. Sample analyses were performed under the same chromatographic conditions as the standards. An example chromatogram of the samples obtained after storing of one of tested materials in water for 1 hour, 24 hours, and 7 days is presented in the Fig. 1. In order to eliminate positively false results, a chromatographic analysis was performed for the matrix (HPLC grade water solution of the Antibiotic Antimicotic preparation) incubated for 1 hour, 24 hours, 7 days or 30 days, under the conditions described above.

Table 1. Distribution of mobile phase concentrations in the chromatographic system

time (min.)	% H_2O	% acetonitryle
0	70	30
20	30	70
15	0	100
28	30	70
30	70	30

Table 2. Reagents used for standard solutions of identified chemical compounds

Chemical compound	Short name	Producer	CAS number volume / weight
Triethylene glycol-dimethacrylate	**TEGDMA**	Sigma Aldrich, USA	261548 – 250ml
ethylene glycol dimethacrylate	**EGDMA**	Sigma Aldrich, USA	335681 – 5ml
Urethane dimethacrylate	**UDMA**	Sigma Aldrich, USA	436909 – 100ml
Bisphenol A	**BPA**	Sigma Aldrich, USA	239658 – 50g

Methods of statistical analysis. For testing of statistical hypotheses, the significance level of p = 0.05 was assumed, and – where the choice was up to the researcher – a two-sided critical region. For continuous variables, the following were calculated: size, arithmetic mean, standard deviation, median, minimum value and maximum value. The basic tool for analysis of means was the single-factor analysis of variance (ANOVA). A normal distribution was assumed. The homogeneity of variance was tested with the Brown-Forsythe test. For multiple testing, the Newman-Keuls test and the Tukey's test were used.

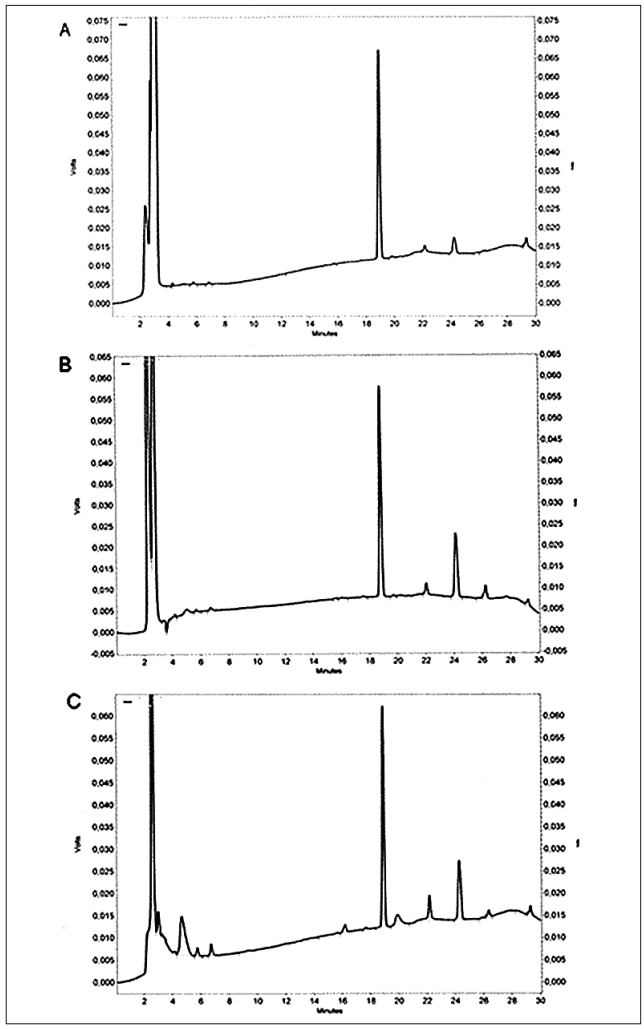

Figure 1. Chromatograms of the samples obtained after storing of one of tested materials (Filtek Z550) in water for 1 hour (a), 24 hours (b), and 7 days

RESULTS AND DISCUSSION

A comparative analysis of concentrations of BPA released into the water solution during the experiment demonstrated that a statistically significant (p = 0.05) bisphenol A concentrations in the assessed eluates was observed after 24 hours and after seven days of incubation. After one hour of observation, the highest bisphenol A concentration was measured in Gradia Direct Anterior eluates, at 0.576 µg·cm^{-3} (Tab. 3). The same composite released the most BPA in subsequent observation periods, with the compound's concentrations of 1.809 µg·cm^{-3} after 24 hours, 1.546 µg·cm^{-3} after seven days, and 0.702 µg·cm^{-3} after 30 days.

The lowest BPA concentrations were observed in eluates from the Filtek Z550 composite: 0.023 µg·cm^{-3} after one hour, 0.075 µg·cm^{-3} after 24 hours, 0.079 µg·cm^{-3} after seven days, and 0.014 µg·cm^{-3} after 30 days.

In the case of TEGDMA released from dental composites, the statistical analysis did not indicate any statistically significant (p = 0.05) differences between the compound's

Table 3. Mean concentrations of BPA released from individual composite materials after consecutive storage periods

Material	1 hour concentration		24 hours concentration		7 days concentration		30 days concentration	
	BPA (μg · cm^{-3})	std	BPA (μg · cm^{-3})	Std	BPA (μg · cm^{-3})	std	BPA (μg · cm^{-3})	std
Gradia Direct Anterior	0.576	0.129	1.809	0.326	1.546	0.272	0.702	0.258
Arkon	0.061	0.020	0.167	0.028	0.122	0.037	0.047	0.046
Filtek Z550	0.023	0.013	0.075	0.016	0.079	0.009	0.014	0.006
Herculite XRV	0.040	0.019	0.306	0.065	0.650	0.144	0.091	0.055
Tetric EVO Ceram	0.044	0.027	0.138	0.028	0.142	0.042	0.101	0.010
Charisma	0.086	0.038	0.285	0.105	0.137	0.083	0.081	0.015

Table 4. Mean concentrations of TEGDMA released from individual composite materials after consecutive storage periods

Material	1 hour concentration		24 hours concentration		7 days concentration		30 days concentration	
	TEGDMA (μg · cm^{-3})	std	TEGDMA (μg · cm^{-3})	std	TEGDMA (μg · cm^{-3})	std	TEGDMA (μg · cm^{-3})	std
Gradia Direct Anterior	0.000	0.000	0.002	0.918	0.000	0.000	0.000	0.000
Arkon	1.913	0.156	2.913	0.004	2.234	0.853	2.951	1.477
Filtek Z550	0.075	0.016	0.058	0.392	0.053	0.026	0.039	0.013
Herculite XRV	2.045	0.939	2.139	0.013	3.375	0.849	2.032	0.657
Tetric EVO Ceram	0.000	0.000	0.000	0.820	0.018	0.041	0.000	0.000
Charisma	2.845	0.507	5.348	0.000	8.112	2.271	6.458	1.328

concentrations observed at particular points in time during the experiment. After one hour of storage in water, the highest mean TEGDMA concentration of 2.845 μg·cm^{-3} was observed in Charisma composite eluates. The compound was not identified in eluates from the Gradia Direct Anterior material (Tab. 4).

As for eluates obtained after 24 hours of observation, the highest concentration of the monomer was identified in Charisma composite samples at 5.348 μg·cm^{-3}. TEGDMA was not observed in the water solution of the Tetric Evo Ceram material. Charisma demonstrated the highest TEGDMA concentrations also at the subsequent points in time during the experiment, and the compound's concentration after seven and 30 days equalled 8.112 μg·cm^{-3} and 6.458 μg·cm^{-3}, respectively. After seven days of sample storage in water, the compound was not identified in eluates from Gradia Direct Anterior, and after 30 days – in eluates from Gradia Direct Anterior and Tetric Evo Ceram (Table 4).

Similarly to the above chemical compound, UDMA release was statistically significantly the lowest (p = 0.05) after one hour of composite's storage in the water environment, with the 0.018 μg·cm^{-3} concentration. UDMA concentrations in eluates after 24 hours, seven days and 30 days were not statistically significantly different (p = 0.05) between each other. During all observation periods, the highest UDMA concentrations were observed in Gradia Direct Anterior eluates, at 0.758 μg·cm^{-3} after one hour, 4.402 μg·cm^{-3} after 24 hours, 5.232 μg·cm^{-3} after seven days and 4.118 μg· cm^{-3} after

Table 5. Mean concentrations of UDMA released from individual composite materials after consecutive storage periods

Material	1 hour concentration		24 hours concentration		7 days concentration		30 days concentration	
	UDMA (μg · cm^{-3})	std	UDMA (μg · cm^{-3})	std	UDMA (μg · cm^{-3})	std	UDMA (μg · cm^{-3})	std
Gradia Direct Anterior	0.758	0.112	4.402	0.704	5.233	1.604	4.118	0.960
Arkon	0.149	0.018	0.475	0.040	0.470	0.121	0.272	0.126
Filtek Z550	0.470	0.177	1.218	0.261	1.724	0.379	0.576	0.139
Herculite XRV	0.051	0.008	0.020	0.024	0.010	0.014	0.002	0.005
Tetric EVO Ceram	0.403	0.129	2.188	0.189	2.545	0.586	1.202	0.116
Charisma	0.018	0.024	0.016	0.007	0.008	0.012	0.000	0.000

30 days of sample storage (Tab. 5). The lowest concentration of the monomer was observed in samples of Charisma eluates. After one hour, it equaled 0.018 μg·cm^{-3}, after 24 hours 0.016 μg·cm^{-3}, after seven days 0.008 μg·cm^{-3}, and after 30 days UDMA was not identified in Charisma samples.

After one-hour storage of the studied materials in water, EDGMA secretion was not detected in samples of Trans Bond XT, Gradia Direct and Charisma (0.000 μg·cm^{-3}) materials. The samples obtained from the solution after 24 hours of storage of the composite materials, the highest statistically significant (p = 0.05) concentration of EGDMA was determined in eluates from Herculite XRV (0.226 μg·cm^{-3}) and Charisma (0.442 μg·cm^{-3}) materials. The presence of this compound was not detected in solutions obtained after incubation in water of Trans Bond XT, Arkon and Filtek Z550 materials. As for eluates obtained after 7-day storage of composite materials in water, the highest statistically significant (p = 0.05) concentrations of EDGMA was observed in the case of Charisma at 2.334 μg·cm^{-3}. The eluates obtained after 30-day incubation, the highest statistically significant (p = 0.05) concentration of EGDMA was observed for the Charisma material and it equaled 1.614 μg/ml.

EDGMA concentrations in solutions of the evaluated in materials determined at particular time intervals are presented in Table 6.

Studies published in available literature confirm the phenomenon of methacrylate monomers release from composite materials used in the reconstruction of hard tissues of the teeth [21].

The results of the study confirm the release of chemical compounds, which are potentially harmful to health, from dental composite materials used for the reconstruction of hard tissues of teeth. BPA, TEGDMA, UDMA and EDGMA eluted from dental polymers indicate a cytotoxic potential and cause damage to the structure of nucleic acids in cells from tissue cultures; they also impair immune response of lymphocytes, which is confirmed by studies based on cytotoxicity tests [22] and on assessment of mutagenic potential [23]. In the oral environment they cause inflammatory reaction of tooth pulp, leading to its necrosis, and induce topical allergic reactions from oral mucous membrane. Bisphenol A, released from composite materials assessed in the presented study, is a compound which has an adverse effect on metabolic processes and cell structures. Parahormonal action of bisphenol A, which activates estrogen receptors, has been confirmed,

Table 6. Mean concentrations of EDGMA released from individual composite materials after consecutive storage periods

Material	1 hour concentration		24 hours concentration		7 days concentration		30 days concentration	
	EDGMA ($\mu g \cdot cm^{-3}$)	std	EDGMA ($\mu g \cdot cm^{-3}$)	std	EDGMA ($\mu g \cdot cm^{-3}$)	std	EDGMA ($\mu g \cdot cm^{-3}$)	std
Gradia Direct Anterior	0,000	0,000	0,023	0,016	0,016	0,005	0,030	0,009
Arkon	0,007	0,002	0.000	0.000	1,009	0,903	0,949	0,401
Filtek Z550	0,000	0,001	0.000	0.000	0,229	0,088	0,323	0,108
Herculite XRV	0,001	0,002	0,226	0,070	0,994	0,162	0,886	0,330
Tetric EVO Ceram	0,001	0,003	0,054	0,056	0,801101	0,133	1,228	0,282
Charisma	0.000	0.000	0,442	0,081	2,334274	0,689	1,614	0,274

as well as its unfavourable impact on differentiation and maturing of zygotes in laboratory animals [11].

Both the European Commission [24] and US Food and Drug Administration [25] have introduced legal changes aiming at limiting baby exposure to BPA released from feeding bottles. Dental materials, besides containers for food storage, constitute one of the main sources of exposure to bisphenol A.

Therefore, it seems necessary to clarify the safety issues related to the use of composite materials in dentistry, as they release methacrylate monomers into the external environment, especially bisphenol A, and especially during the treatment of pregnant women and children. The characteristics of medical products declared by theirs producers should be confirmed by both laboratory and clinical trials to ensure their safety during treatment procedures.

CONCLUSIONS

1. The examined composites used for the reconstruction of hard tissues of teeth remain chemically unstable after polymerization, and release potentially harmful substances in conditions of the presented study.
2. The dynamics of the releasing of potentially harmful substances is correlated with the period of sample storage in water.

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