Organic leachables from resin-based dental restorative materials

Characterization by use of combined gas chromatography - mass spectrometry

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Dissertation for the degree of philosophiae doctor (PhD)

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Department of Oral Sciences – Dental Pharmacology

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and

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Abstract

During the last decade resin-based dental restorative materials have replaced amalgam as the first choice dental filling material. Resin-based dental restorative materials are complex polymers containing a variety of monomers and filler particles, as well as initiators, activators, stabilizers, plasticizers and other additives. Several studies have shown that many of the ingredients are leaching from the materials, even after adequate polymerization. It is known from in vitro studies that some of the compounds in the resin-based materials have cytotoxic, genotoxic or estrogenic potential. Allergenic effects in patients and dental personnel have also been reported.

The aim of this study was to identify and quantify substances released from various types of resin-based dental restorative materials. Specimens were polymerized according to protocols from the producers and submerged in different solvents. To characterize a maximum elution potential, ethanol was used as an immersion media. Furthermore, immersion in Ringer's solution and saliva was used to mimic clinical elution conditions.

A combined Gas Chromatography - Mass Spectrometry (GC-MS) technique was used for the identification and quantification of eluates. The majority of eluting substances from the polymeric matrix are organic substances with low molecular weight, which are well suited for analysis by GC-MS. Tailor-made internal standards for HEMA and TEGDMA were synthesized for the quantification procedure.

We have identified and quantified a number of compounds from several materials. Significant differences regarding type and amount of leachables between the materials are observed.

List of publications

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

Paper I:

Michelsen VB, Lygre H, Skålevik R, Tveit AB and Solheim E. "Identification of organic eluates from four polymer-based dental filling materials", Eur J Oral Sci. 2003; 111 (3):263-271.

Paper II:

Michelsen VB, Moe G, Skålevik R, Jensen E, Lygre H. Quantification of organic eluates from polymerized resin-based dental restorative materials by use of GC/MS. J Chromatogr B Analyt Technol Biomed Life Sci. 2007 May 1;850 (1-2):83-91.

Paper III:

Michelsen VB, Moe G, Strøm MB, Jensen E and Lygre H. Quantitative analysis of TEGDMA and HEMA eluted into saliva from two dental composites by use of GC/MS and tailor-made internal standards. Dent. Mater. Article in press, available online September 2007.
doi: 10.1016/j.dental.2007.08.002

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ABBREVIATIONS

ADA	American Dental Association
amu	Atomic mass units
BHT	Butylated hydroxytoluene
BI	Benzene iodide
Bis-DMA	Bisphenol A dimethacrylate
Bis-EMA	Ethoxylated Bisphenol-A-dimethacrylate
Bis-GMA	Bis-glycid-dimethacrylate
Bis-HPPP	2,2-bis[4 (2,3-hydroxy-propoxy)phenyl]propane
bp	Boiling point
BPA	Bis-phenol-A
BPS	Butylphenyl sulfone
BSA	Benzenesulfinic acid sodium salt
BTS	S-phenyl benzenethiosulfonate
9-F	9-Fluorenone
BZ	Benzil
CAS	Chemical Abstracts Service
CQ	Camphoroquinone
DC	Degree of Conversion. DC is defined as the percentage of C=C double bonds that disappear during polymerization
DEG	Diethylene glycol
DMABEE	Benzoic acid, 4- (dimethylamino)-, ethyl ester
DMPA	2,2-dimethoxy-2-phenyl-acetophenone
DPICl	Diphenyliodonium chloride
EEA	2-Ethoxyethyl acrylate
EGDMA	Ethyleneglycol dimethacrylate
FTIR	Fourier Transform Ifrared Spectroscopy
GC	Gas Chromatography
HC	2 (3)-endo-hydroxyepicamphor
HEMA	2- Hydroxyethyl methacrylate
HMBP	Oxybenzone
HPLC	High-Performance Liquid Chromatography
HPMA	2-Hydroxypropyl methacrylate
HQ	Hydro quinone
I.S.	Internal standard
IPM	Isopropyl myristate
IR	Infrared

ISO	The International Organization for Standardization
IUPAC	International Union of Pure and Applied Chemistry
LC/PB/MS	Liquid Chromatography/Particle Beam Interface/Mass Spectrometry
m/z	Mass-to-Charge Ratio
MeOH	Methanol
MEHQ	Mequinol
ml	Milliliter
MMA	Methyl methacrylate
MS	Mass Spectrometry
MSDS	Material Safety Data Sheet
MW	Molecular Weight
ORMOCERS	Organically Modified Ceramics
R^2	Coefficient of determination
RMGIC	Resin modified glass ionomer cement
Rpm	Rounds per minute
RT	Retention Time
SD	Standard deviation
SEM	Scanning electron microscope
SMP	Sulfone, methyl phenyl
SPMS	Benzenethiosulfonate
SPSS	Statistical Package for the Social Science
TCB resin	Tetracarbonsyre-hydroxyethyl methacrylat-ester
TEEGDMA	Tetraethyleneglycol dimethacrylate
TEGDMA	Triethyleneglycol dimethacrylate
TEGMA	Triethyleneglycol methacrylate
TIN P	Tinuvin P
TMPTMA	Trimethylolpropane trimethacrylate
TPSb	Triphenyl Antimony
UDMA	Urethane dimethacrylate
UV	Ultraviolet

1. Introduction

Resin-based dental restorative materials formulated as composites were introduced in dentistry in the early 1960s, subsequent to Michael Buonocore's introduction of the acid-etch technique and Dr. Rafael Bowen's patent on Bis-GMA (1). The original composites were two-component, self-curing materials. More operator friendly materials for light-induced polymerization became commercially available in the late 1970's. In an effort to achieve aesthetic, biocompatible and durable repair of teeth, new materials have been formulated and made accessible for dental treatment at an increasing rate. Composite is today the first choice restorative material in several countries, including Norway, Sweden and Finland. According to recent Scandinavian studies, the proportion of tooth-colored materials chosen for restorative therapy varied between 65- 94 % (2-6).

The reasons why resin-based dental fillings have outnumbered amalgam fillings during the last decades are many. A growing anxiety regarding adverse effects from the mercury content of amalgam, and an environmental concern regarding the toxic potential of the waste disposal of this material, has contributed to this trend. A report on dental restorative materials published in Norway in 1998 (7), was followed by new guidelines from the Directorate for Health and Social Affairs, Norway (8). The guidelines state that amalgam should not be the first choice material when placing dental restorations. A demand for aesthetic dentistry and a greater emphasize on conservation of tooth structure, are other reasons why resin-based materials are preferred. Due to the dental adhesive technique used in combination with the resin-based materials, cavity preparation is minimized, thereby preserving tooth substance and reducing possible pulp damage.

Resin-based dental restorative materials are complex polymers consisting of reinforcing filler particles embedded in an organic, resin-based matrix. Usually this

matrix is based on methacrylate chemistry. In addition, a binding system between fillers and resin is required. The organic matrix may contain a variety of monomers, as well as initiators, activators, stabilizers, plasticizers and other additives. The main organic ingredients are monomers which during polymerization are cross linked with less viscous monomers to create a rigid polymer network. As the cross-linking proceeds, circulation inside the network becomes limited, and a significant amount of monomers and additives remain trapped but unbound in the cured material (9). A complete polymerization is therefore not possible to achieve. Due to elution, erosion and degradation, unbound ingredients and degradation products may escape from resin-based materials into the oral cavity (10), or diffuse through dentin and into the pulp (11, 12).

From *in vitro* studies it is known that constituents and degradation products in resin-based materials may have cytotoxic (13-18), genotoxic (19-22) or estrogenic (10, 23-25) potential. Allergic reactions have been shown in several *in vivo* reports (26-29). A large number of people are exposed to dental restorative materials; dental biomaterials are among the most extensively used artificial materials in man. Dental restorative filling materials are intended for long-term service in the oral cavity, and when additional treatment is required, a replacement or repair with resin-based material is often preferred.

Consideration of the stability of the materials *in vivo*, and the character of substances released, is essential to evaluate the safety of a dental material. Different materials may have different potentials for causing adverse effects. In assessment of biocompatibility both quantitative and qualitative information concerning eluates has to be considered. Information from the manufacturers about the products' ingredients given in the Material Safety Data Sheets (MSDS), is often incomplete and sometimes misguiding (30). In addition, degradation products formed during and after curing, and impurities from the production process, may be present in the polymerized material, such as TPSb and Bis-phenol A (31-33) (Table 1). Knowledge about the

composition of the products is essential if an adverse reaction associated with dental products, is suspected or revealed. Such information may give an opportunity to specifically select an appropriate filling material in an individual situation, i.e. when an allergic reaction to an ingredient is known.

Several analytical methods for characterization of released substances from the resin-based dental restorative materials have been applied (31-32, 34-40). Among the most extensive reports identifying eluting substances, are studies from 1994 and 1998 using GC-MS and LC/PB/MS (Liquid Chromatography/Particle Beam/Mass Spectrometry) techniques (32, 39, 40). Elution studies of resin-based dental restorative materials provide valuable information about compounds that might be released from the materials. To assess the risk eluates pose to the human body, we need reliable methods to determine what is eluted, how much is eluted, for how long and at what rate the elution takes place. The purpose of this thesis was to add knowledge about the identity of substances that are eluting form the resin-based dental restorative materials, and develop and apply reliable methods for the quantification of these substances.

1.1 General background

1.1.1 Resin-based dental restorative materials

Several types of resin-based materials are commonly used in dentistry; restorative or filling materials, luting materials, adhesives, flow materials, root-sealers, prosthodontic and orthodontic materials. Only dental resin-based materials used as restorative filling materials for direct application are discussed here. Polymerization of resin-based materials may either be chemically activated, light-induced or triggered by a dual system. In the thesis the studies are restricted to the materials with light-induced polymerization. However, the analytical methods may be applied for the

analysis of organic, low-molecular weight compounds eluted from other types of resin-based materials.

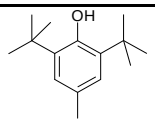
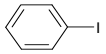
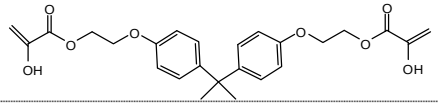
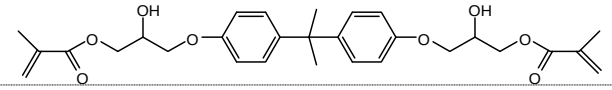
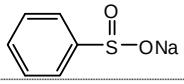
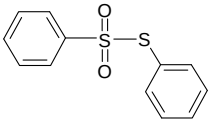
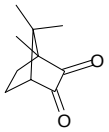
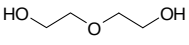
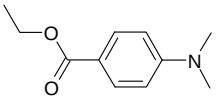
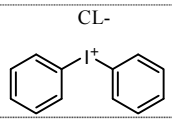
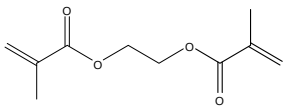
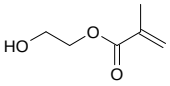
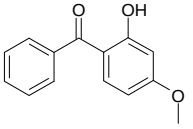
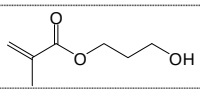
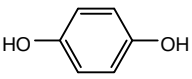
Based on the chemical composition of the matrix, four main categories of resin-based dental restorative materials are commercially available; composites, compomers, resin modified glass ionomer cements and organically modified ceramic materials. The composites contain a monomer system with mainly di-functional methacrylates and filler particles with a coupling agent. Compomers (polyacids-modified resin composites) differ from the composites in that the polymeric matrix is added carboxyl acid modified monomers with the purpose of binding to embedded ion-leachable glass (41). The resin modified glass ionomer cements (RMGIC) are predominately degradable glass particles with polyacids, modified with the addition of polymerizing monomers, such as HEMA (2- Hydroxyethyl methacrylate) (41). Compared to the compomers, the resin phase in RMGIC is present in minor quantity. Another group of materials, which are not investigated in this thesis, are the organically modified ceramic materials (ORMOCERS). In these materials the amount of dimethacrylate monomers has been reduced compared to in the traditional composites, and replaced with polysiloxanes with functionalized methacrylate groups as the main component of the monomer matrix (42). The diverse selection of materials is developed to meet the variety of requirements for restorative materials designed for use in the highly demanding environment in the oral cavity.

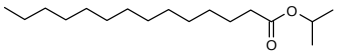
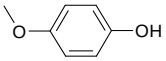
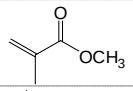
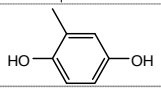
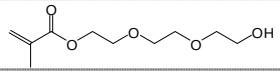
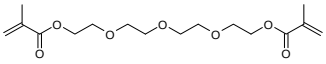
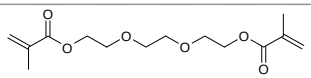
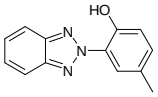
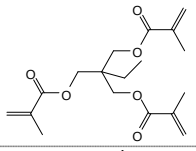
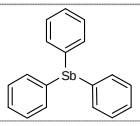
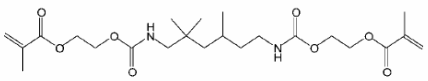
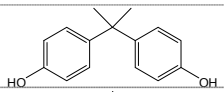
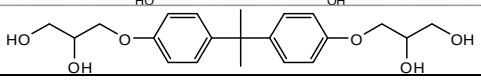
1.1.2 Monomers and degree of cure

The main organic monomers in the resin-based materials are aliphatic or aromatic methacrylates, Table 1. Mono- or dimethacrylates are most commonly used; tri- and oligomethacrylates are less common ingredients. These monomers are bifunctional; i.e. to be able to crosslink the organic matrix into a polymerized network. Traditionally, Bis-GMA (Bisphenol-A-glycid-dimethacrylate) and TEGDMA (triethylglycol dimethacrylate) were used (43). Today, other lower viscosity and more

chemically stable monomers like Bis-EMA (Ethoxylated Bisphenol-A-dimethacrylate) and UDMA (Urethane dimethacrylate) are common ingredients (Table 1). Monomers with low molecular weight, such as TEGDMA, EGDMA (Ethyleneglycol dimethacrylate), TMPTMA (Trimethylolpropane trimethacrylate) and HPMA (2-Hydroxypropyl methacrylate) are used as diluting monomers to decrease the viscosity and increase the cross-linking in the filling materials (44). More cross-linking will, however, increase the polymerization shrinkage, and consequently the type and amounts of monomers must be balanced to the best functionality (43). The obtained cross-linking density after polymerization is described as the degree of conversion (DC). DC is defined as the percentage of C=C double bonds that converts into single bonds during polymerization (44) and may be measured by Fourier Transform infrared spectroscopy (FTIR) or differential thermal analysis (DTA) (45). The resin-based dental restorative materials do not reach full polymerization. At the termination of polymerization, up to 60-70% of the monomer's double bonds may remain unreacted and complete cure (DC = 100%) is never accomplished (46, 47). The percentage of unreacted C=C double bonds after polymerization is only to a degree correlated to the potential of elution of unreacted monomers (9), as most di-functional monomers will react and have at least one methacrylic group covalently bond to the polymer network. An increased content of monomers with a high ability to cross-link, expressed as a high functionality, may increase the degree of conversion (48). It has been estimated that up to 10 % of the monomers have both ends' double bonds intact (residual monomers), free to migrate or elute out of the network (9, 49, 50). Ruggeberg and Craig found that the residual monomer content in the material after curing was correlated to the amount of elution (51). From inadequately cured materials, elution has been found to be considerably higher (52). This may be the reason why cellular toxicity was found to increase as percentage of monomer conversion decreased (53).

Table 1. Organic monomers, additives and degradation products referred to in the thesis.

Compound and chemical formula, CAS number and function	**Structural formula**
BHT $C_{15}H_{24}O$ 128-37-0 Stabilizer	
BI C_6H_5I 591-50-4 Degradation product	
Bis-EMA $C_{27}H_{32}O_6$ 24448-20-2 Monomer	
Bis-GMA $C_{29}H_{36}O_8$ 1565-94-2 Monomer	
BSA $C_6H_5SO_2Na$ 873-55-2 Initiator	
BTS $C_{12}H_{10}O_2S_2$ 1212-08-4 Part of initiator system	
CQ $C_{10}H_{14}O_2$ 10373-78-1 Initiator	
DEG $C_4H_{10}O_3$ 111-46-6 Degradation product	
DMA BEE $C_{11}H_{15}NO_2$ 10287-53-3 Co-initiator	
DPICl $C_{12}H_{10}ClI$ 1483-72-3 Initiator	
EGDMA $C_{10}H_{14}O_4$ 97-90-5 Monomer	
HEMA $C_6H_{10}O_3$ 868-77-9 Monomer	
HMBP $C_{14}H_{12}O_3$ 131-57-7 UV-Stabilizer	
HPMA $C_7H_{12}O_3$ 27813-02-1 Monomer	
HQ $C_6H_6O_2$ 123-31-9 Inhibitor	

IPM $C_{17}H_{34}O_2$ 110-27-0	
MEHQ $C_7H_8O_2$ 150-76-5 Inhibitor	
MMA $C_5H_8O_2$ 80-62-6 Degradation product	
MHQ $C_7H_8O_2$ 95-71-6 Inhibitor	
TEGMA $C_{10}H_{18}O_5$ Degradation product	
TEEGDMA $C_{16}H_{26}O_7$ 109-17-1 Monomer	
TEGDMA $C_{14}H_{22}O_6$ 109-16-0 Monomer	
TIN P $C_{13}H_{11}N_3O$ 2440-22-4 UV-Stabilizer	
TMPTMA $C_{18}H_{26}O_6$ 3290-92-4 Monomer	
TPSb $C_{18}H_{15}Sb$ 603-36-1 Catalyst from monomer production	
UDMA $C_{23}H_{38}N_2O_8$ 72869-86-4 Monomer, Several isomers Diurethane dimethacrylate	
Bis-phenol A $C_{15}H_{16}O_2$ 80-05-7 Degradation product	
Bis-HPPP $C_{21}H_{28}O_6$ Degradation product	

1.1.3 Initiators and coinitiators

The monomer system needs chemicals to promote the polymerization reaction. In materials with light induced polymerization, a photo-initiator and a co-initiator are added. Photons from a light source produce an exited state of the initiator, which interacts with the co-initiator, usually benzoic acid, 4- (dimethylamino)-, ethyl ester (DMA BEE), to form free radicals. The free radicals react with the monomers and the conversion by chain growth to polymers is started (41). Several initiators are suitable for the polymerization process. Camphoroquinone (CQ) is by far the most frequently

used initiator, required in typical concentration of 0.2-0.3 weight percent (54). CQ absorbs light within the range of 400 to 500 nm, with the maximum absorbance at around 468 nm, thus curing lamps should emit light with a high intensity in this range. During the polymerization the mobility of single molecules are gradually reduced, which may promote the existence of free radicals several days after initiation (47). Degradation of CQ may be found as HC (2 (3)-endo-hydroxyepichamphor) (39). The color of camphoroquinone is intense yellow and represents therefore an aesthetic problem. Thus in bleach shades and translucent shades other initiators, such as DIPICl (Diphenyliodonium chloride), may be applied.

1.1.4 Inhibitors

A preferred situation is that the polymerization is initiated only when the curing lamp is started. However, heat or day-light may activate the initiator system prematurely. To avoid pre-polymerization in storage, small amounts of stabilizers, such as MEHQ (mequinol) or BHT (butylated hydroxytoluene) (Table 1), are added (9). The inhibitors are molecules with a strong potential to react with free radicals, and is one of the factors deciding the working and setting time for the material. Monomers as raw materials also require a protection against self-polymerization, and are usually stabilized with MEHQ, HQ (hydroquinone) or BHT. Such information may be found in the MSDS accompanying the monomer products. Stabilizers have been found eluted from the resin-based materials (32, 39).

The polymerization process is retarded by oxygen due to oxygen's rapid reaction with free radicals. The surface of a resin-based dental filling is, for this reason, not polymerized optimally, unless a matrix is applied to prevent the oxygen inhibition (55).

After the restoration has been placed and cured in the oral cavity, the restorations may be exposed to UV-light. UV-light induces photochemical reactions with possible

discoloration as a result. By adding small amounts of specific UV-stabilizers the resin matrix is protected against the UV-light. The most common UV-stabilizers used in the resin-based dental materials are HMBP (oxybenzone) (56) and Tin P (Tinuvin P) (57).

In addition to the resin system and fillers, the materials contain pigments for optical modifications and molecules absorbing x-rays, such as barium, aluminum, strontium, zinc, zirconium, ytterbium or yttrium (58, 59). These substances are present to enhance the contrast of the filling materials on radiographic films.

1.1.5 Fillers

Various inorganic materials such as minced quarts, melt glasses and ceramics, and organic prepolymerized resin particles, are used as fillers to improve the mechanical and physical properties and reduce the polymerization shrinkage of the resin-based materials. Because of the fillers' major impact on the materials properties, the most common classification of composites is based on the filler characteristics; the fillers' particle size and size distribution (60). Compomers and resin-reinforced glass ionomer cements are generally not further classified. Composites might be classified as traditional or microfilled or a hybrid of these (41). As new formulations of composites are made accessible, the classification has been gradually modified and subgroups and overlapping between groups has appeared. Anusavice classified traditional composites as materials with particle size 1-50 μm, a microfilled composite with particles of average size 0.04 μm, and hybrids of these with small particle size 0.1-20 μm and micro-particles 0.04 μm (41). The term "nanofillers" or "nano" scale particles have been introduced by producers the last years, although these particles may not necessarily be different from micro-particles. These composites have particles with size up to 100 nm (0.1 μm). The nano fillers may be arranged separately but tend to condensate into clusters or agglomerates (44). In general microfilled composites have superior polishing properties which enhance the aesthetic properties, whereas hybrid composites have excellent stress-bearing

properties. Nanofillers have been developed to combine the aesthetic properties required and the mechanical properties necessary for stress bearing restorations in premolars and molars (61). Due to erosion and degradation the filler particles may be released to the surrounding environment (59).

1.1.6 Coupling agents

To enhance the bonding between the filler particles and the resin matrix, the fillers are coated with a coupling agent. This coupling agent (silane) has a functional group that is able to form covalent bonds to the methacrylates in the resin phase during the process of polymerization. Organosilanes such as γ-methacryloxypropyl trimethoxysilane is most commonly used. The strong binding between the fillers and the resin reduces the erosion and elution of the fillers. In addition, the silane enhances the stress transfer within the material and thus minimizes the initiation of fractures (62).

1.1.7 Degradation and elution

Substances and particles are released from the dental filling materials by a process of elution or by degradation, either as chemical or physical disintegration and dissolution. (63, 64). Degradation may reduce the longevity of a restoration; however, of greater concern is the possibility of biological adverse effects caused by the eluates.

The unbound substances from the cured material released into the immersion media are called eluates or leachables (65). Elution may take place from the bulk and from the surface of the material. A strong correlation between the surface area of the specimens and the amounts of eluted TEGDMA has been demonstrated (66). The degree of elution from the surface is influenced by the conditions at the moment of polymerization. Oxygen inhibits the polymerization of the surface layer, and if

present, the amounts of leachables may increase (67). Mohsen showed that removing the oxygen inhibiting layer reduced the cytotoxicity of the material (55). This oxygen-inhibited layer will not be present if curing takes place in an oxygen free environment. Enhanced release of compounds not bound in the resin-based materials is suggested after swelling caused by uptake of water or other solvents (68).

1.2 Biocompatibility of resin-based restorative materials

Biocompatibility is defined as "the ability of a material to perform with an appropriate host response in a specific application" (69). To screen and summarize if resin-based dental restorative materials are biologically acceptable, a set of toxicity test may be used (70). The tests may involve test for systemic toxicity, cytotoxicity, sensitization, genotoxicity, mutagenicity, carcinogenicity, reproductive toxicity, irritation and reactivity. Toxicokinetic studies shall be considered when a restoration material release substantial quantities of potentially toxic or reactive degradation products into the body during clinical use (71).

1.2.1 Potential adverse effects

Any material applied in the body may give a local or a systemic biological effect, primarily by substances released from the materials (41). Possible biological reactions has been separated into different categories, in reality the strict boundaries between the categories cannot be drawn (72). General toxic reactions in dental patients are considered not likely to occur due to the low exposure to components derived from disintegration of dental materials (73). However, *in vitro* studies have shown that some of the substances show cytotoxic effects at quantities which may be of concern (13-18, 72). Genotoxic (19-22) and estrogenic effects from ingredients of resin-based materials has been found in several studies (10, 23-25). Adverse effects described as allergic reactions in patients and dental personal have been increasingly reported during the last ten years (26-29, 74).

The cytotoxic potential of several components in a dose-dependent pattern has been clearly documented, with a diversity of the potencies of these substances, Table 2 (13-18, 75). It is difficult to assess the cytotoxic risks that these materials pose *in vivo.* Eluted components from dental restorative materials have been found in sufficient concentrations *in vivo* to be regarded as cytotoxic (14, 76, 77).

It has repeatedly been demonstrated *in vitro* that TEGDMA and HEMA cause gene mutations (20, 21, 78). Investigations of possible genotoxicity of several compounds known as ingredients or degradation products from resin-based dental materials, characterized butylated hydroxytoluene (BHT), camphoroquinone, (CQ), triphenyl antimony (TPSb), benzil (BZ), benzophenone (HMBP), and 9-fluorenone (9-F) , Bis-GMA and UDMA to be genotoxic as well (19, 79). The interaction between monomers and DNA is not clearly understood, but it is assumed that the induction of DNA damage may follow different pathways for the different monomers (72).

Ingredients or degradation products from resin-based materials used in dentistry, has been shown to exhibit estrogenic activity *in vivo* (80) and *in vitro (23)*. Compounds with such properties include Bis-phenol-A (BPA) and Bis-phenol A dimethacrylate (Bis-DMA). Bis-phenol A was first detected in saliva by Olea et al. (10), although controversy exists about this report regarding both the method and the interpretation of the results (81). Release of Bis-phenol-A was later reported to be found in saliva after placing fissure sealants (33, 82). *In vitro* studies confirmed the elution of Bis-phenol-A (83-85) from sealants and restorative materials, whereas in other *in vitro* studies the presence of Bis-phenol-A could not be verified (86, 87). The previous mentioned studies suggest that Bis-phenol-A is a degradation product from Bis-DMA or present as an impurity from the synthesis of Bis-DMA and Bis-GMA. Most likely Bis-GMA will not be degraded to Bis-phenol-A (83, 88). The concentration in saliva is believed to be low or not detectable and the short-term risk of estrogenic effects from treatments using Bis-phenol-A-based resins is thus considered as insignificant, although, long-term effects are not clarified (82, 89, 90). Several other substances

have been tested for estrogenic activity (91). Wada et al. reported that the following substances demonstrated such activity; the photo stabilizer, 2-hydroxy-4-methoxy-benzophenone (HMBP), the photo-initiator, 2,2-dimethoxy-2-phenyl-acetophenone (DMPA), and the inhibitor, butylated hydroxytoluene (BHT). It was further concluded that *in vivo* effects were not likely to occur with the low level of concentrations detected (24). On the other hand, long term implications of the release of these substances have not yet been investigated.

Allergy appears to be the primary risk of biological adverse effects from the resin-based dental restorative materials. The prevalence of allergenic effects caused by resin-based materials among patients is low (74). A higher risk of developing occupational allergic contact dermatitis is found among dental personnel partly due to exposure to the methacrylate materials (27, 29). Studies show that these monomers penetrate easily though the gloves, thus gloves are not effective in protection against the contact with monomers (92). Reports on asthma caused by inhalation of methacrylates have also been published (93). Type IV delayed hypersensitivity or contact dermatitis are the most common allergenic reactions in relation to dental materials (27), although, Type I allergy reactions do occur infrequently (REF). Methacrylate monomers are regarded as sensitizers, with weak to non-sensitizing capacity (94). The strongest chemical allergens related to Type IV reactions are often of low molecular weight (MW < 500) and chemically reactive substances, which have the ability to bind to host proteins and result in immune responses (73). Consequences may be tissue damage with clinical manifestations and symptoms. A suspected agent may be confirmed as cause for sensitization by use of epicutan patch testing. However, a positive sensitivity reaction to a substance after patch testing is not necessarily correlated to the outbreak of a reaction associated with the use of a dental restoration containing the sensitizer. A positive response of sensitization after exposure to the oral mucosa is more difficult to accomplish than after exposure to the skin, due to the anatomical and physiological differences between these tissues. Patch

test for oral mucosa does exist, but the concentration of sensitizer needs to be 5-12 times higher than for an epicutan patch test (95) and for practical purposes skin test are commonly used. Related compounds may produce possible cross reactions. Between methacrylates cross reaction have been described (96).

Table 2. Results from selected reports describing cytotoxic, genotoxic, estrogenic or allergenic potential for several eluates. Monomers not detected in the studies are marked with #.

Eluate	**Molecular formula**	***In vitro* observed effects**			
		Cytotoxic	**Genotoxic**	**Estrogenic**	**Allergenic**
HEMA	$C_7H_{12}O_3$	(certain conditions) (14) uncured (97) (1.77mM) (13) (17)			(26, 98-100)
BI	C_6H_5I	0.047mM (13)	(19)		
CQ	$C_{10}H_{14}O_2$	(2.17mM) (13) (16)	(19, 79)		
BHT	$C_{15}H_{24}O$	(0.16mM) (13)	(19)	(24)	
EGDMA	$C_{10}H_{14}O_2$	(0,46mM) (13)			(26, 101)
DMA BEE	$C_{11}H_{15}NO_2$	(1.22mM) (13)			
TEGDMA	$C_{14}H_{22}O_6$	(0.1- 1.5 mM) (13) (15, 17, 102-105)	(21, 22)		(26, 99, 101)
HMBP	$C_{14}H_{12}O_3$	(0.44mM) (13)	(19)	(24)	(106, 107)
TIN P	$C_{13}H_{11}N_3O$				(108, 109)
TPSb	$C_{18}H_{15}Sb$	(0.09mM) (13)	(19)		
#Bis-GMA	$C_{29}H_{36}O_8$	(>0,08mM) (15) (110, 102, 105)	(19, 111)		(99)
#UDMA	$C_{23}H_{38}N_2O_8$	(0,06mM) (15) (102)	(19)		(99)

1.3 Regulations and labelling

Dental materials are regarded as Medical Devices according to the European Council Directive (112). Within Europe the dental materials should have a premarketing approval and bear the CE mark. The CE marking ensures and declares that the actual products meet the general requirements of this Directive. The Directive states:

> "*The devices must be designed and manufactured in such a way that, when used under the conditions and for the purposes intended, they will not compromise the clinical condition or the safety of patients, or the safety and health of users or, where applicable, other persons, provided that any risks which may be associated with their use constitute acceptable risks when weighed against the benefits to the patient and are compatible with a high level of protection of health and safety*".

It is also declared that the devices must be designed and manufactured in such a way to reduce to a minimum the risk posed by substances leaking from the device.

Products sold in Norway, Sweden, Denmark and Island containing chemicals that may pose a health risk to the patient or dental personnel should in addition be marked according to the European regulation for hazardous ingredients and accompanied by a Material Safety Data Sheet (113). As a voluntary assessment, products may be evaluated to ISO standards. Polymer-based filling materials may be tested according to methods and requirements in ISO 4049 (113, 114). In this standard the principal organic components are required to be specified. Biological evaluation of medical devices is considered in ISO 10993, describing standards for characterization of materials, there among the chemical composition and potential leachables (115). Other voluntary evaluation programs for safety and product efficiency exist, such as ADA "Seal of Acceptance" ("ADA Accepted").

1.4 Analytical methods

1.4.1 Chromatography

Chromatography is a family of different separation techniques that are able to resolve a complex sample into its individual components. There are two major subdivisions of chromatography, gas chromatography (GC) (Fig. 1) and liquid chromatography (LC). In most cases a chromatography column (Fig. 2) is used to obtain the separation of the different compounds. Separation is achieved when different compounds have different distribution between the stationary phase (inside the column) and the mobile phase (flows through the column), Fig. 4. In GC the mobile phase is a gas; in LC the mobile phase is a liquid. In both GC and LC there is a large selection of different stationary phases. As indicated, one characteristic feature of chromatographic separation is differential migration of various analytes in the original sample. But there is also a spreading along the column of the molecules of each individual analyte and the result is characterized as band broadening. These processes are in general much less pronounced in GC than in LC and explain why modern GC is superior to LC with respect to give narrow peaks and good separations. In most cases the result of a chromatographic separation is presented in a graph called a chromatogram (Fig. 1).

1.4.2 GC-Instrumentation

A standard GC consists of an injector, the separation column and a detector (Fig. 1).

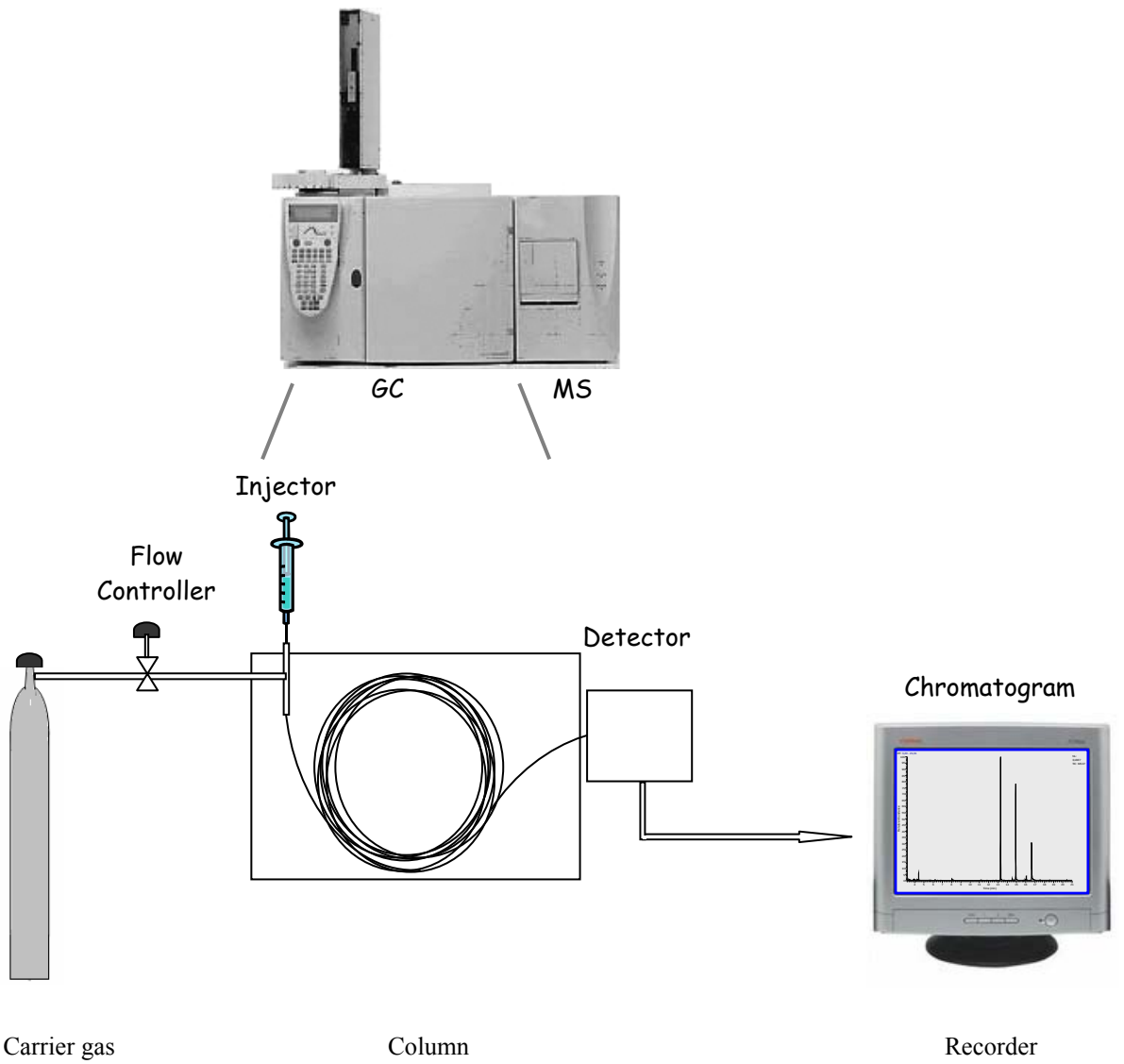


Figure 1. A schematic drawing of a Gas Chromatograph

Different gases can be used as the mobile phase. Most commonly used is nitrogen (N_2), helium (He) or hydrogen (H_2). With capillary columns He is the best compromise between maximum resolution, safety and speed of analysis. Samples can be introduced to the GC via a number of different injectors. The most popular injector is the Flash Vaporizer. By use of high temperature the solvent and the sample molecules instantaneously converts to gas phase and are transported to the separation

column. A disadvantage with this type of injector is that some compounds can disintegrate in the injector due to the high temperatures used. Two major classes of GC-columns are available, packed columns or capillary columns. Packed columns typically are 2 m long with an internal diameter of 2 - 4 mm, and are packed with small porous particles. The stationary phase is a thin film on the surface of these particles. The most commonly used capillary columns are the Wall Coated Open Tubular (WCOT) columns. Typically lengths are 25 - 30 m and the internal diameter is usually 0.25 or 0.32 mm. The stationary phase for a capillary column is coated as a thin film on the inner wall of the column (Figs. 3 and 4). The separation power of a chromatographic column is proportional to the square root of the column's number of theoretical plates – N. Typical values for packed columns are much lower ($N \approx 3\,000$) than typical values for capillary columns ($N \approx 150\,000$). Much better separation can therefore be obtained with capillary columns compared to packed columns. GC-instruments can be equipped with a number of different detectors, such as Flame Ionization Detector (FID), Thermal Couple Detector (TCD) and Electron Capture Detector (ECD). FID and TCD are general detectors, whereas ECD detects only halogen containing compounds, conjugated carbonyls, nitriles and a few other classes of compounds. The GC can be combined with a mass detector (MS). The great advantage of a combined GC-S is that the MS can be used both as a general detector (when used in full scan mode), and as a selective detector (when used in Selected Ion Monitoring –SIM- mode).

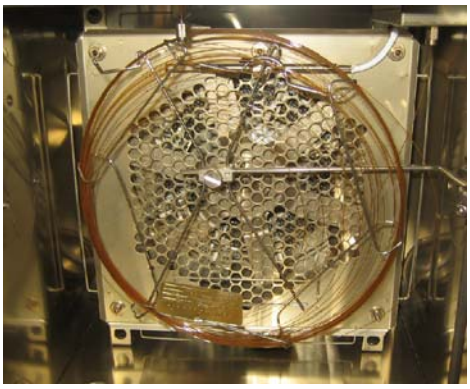


Figure 2. The column

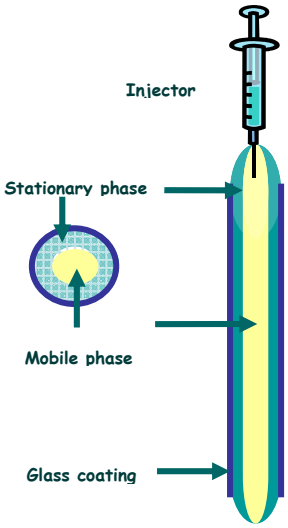


Figure 3.
Cross- and longitudinal section through the column

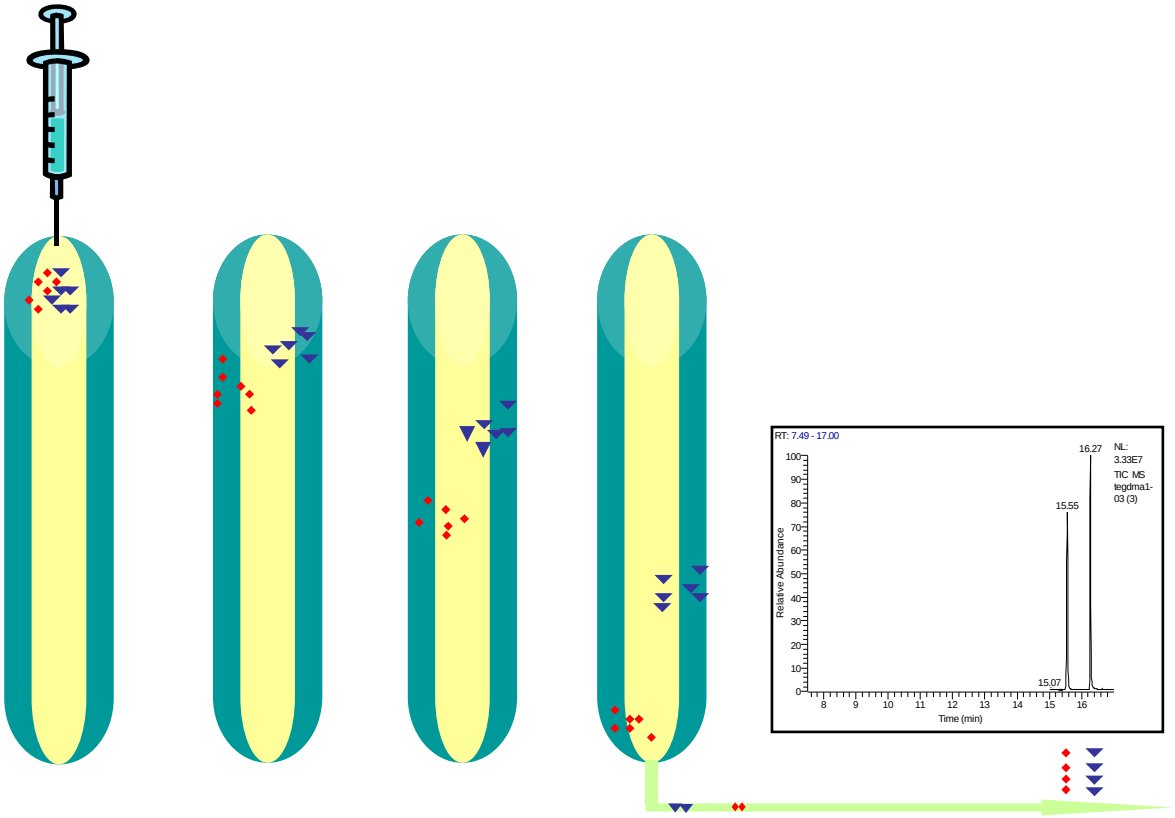


Figure 4. Two substances are injected into the column. The blue compound has a higher affinity for the stationary phase, and moves more slowly through the column, thus the substances are separated. The separated substances are recorded in the chromatogram.

1.4.3 Mass Spectrometry

The technique of mass spectrometry (MS) involves the separation of ions in gas phase according to their mass to charge (m/z) ratio, Fig. 5. The typical MS-instrument consists of an inlet system, an ion source, a mass to charge separation unit (mass filter) and a detector (Figs. 5, 6). In general the mass spectrometer can be operated either in full scan mode (all ions produced in the ion source will be detected and recorded) or in SIM- mode (only one or only a few ions are detected and recorded). When analyzing unknown compounds and using the MS in the full scan mode, the unknown compound can be identified either by comparing the obtained spectrum with library-spectra or by elucidating the structure from the appearance of the obtained spectrum. When using the MS in the SIM-mode in quantitative analysis of known compounds, very selective and sensitive analytical methods are achieved.

A large number of different inlet systems, ion sources and mass filters are available. The instrument used in this study is a GC-MS with an electron impact (EI) ion source and a quadrupole mass filter. Dempster described the EI source already in 1918 (116). Neutral gas phase analytes entering the EI-source are bombarded with high energy electrons (70 eV) and an electron is expelled from the analyte. The charged analytes are directed through electronic lenses into the mass filter. An advantage of EI ionization is that the appearance of the spectrum of a given compound is almost instrument independent, i.e. spectra from two different instruments of the same compound are similar and make it easy to build up extensive libraries.

The quadrupole mass filter consists of four metallic rods (20-30 cm long, 1 cm in diameter) arranged parallel and symmetrical along the axis. The analytes travel from the ion source to the detector (Fig. 6). In a given time, two rods in a diagonally pair has a charge, e.g. +, whereas the other diagonal pair has an opposite charge (-). Subsequently the charge situation is changed to the opposite. By controlling the charge, the voltage and the frequency of the changes, all ions (or only selected ions)

will be directed towards the detector. The recorded ions from the detector are displayed with mass/charge ratio to relative abundance of ions in a mass spectrum (Fig. 5).

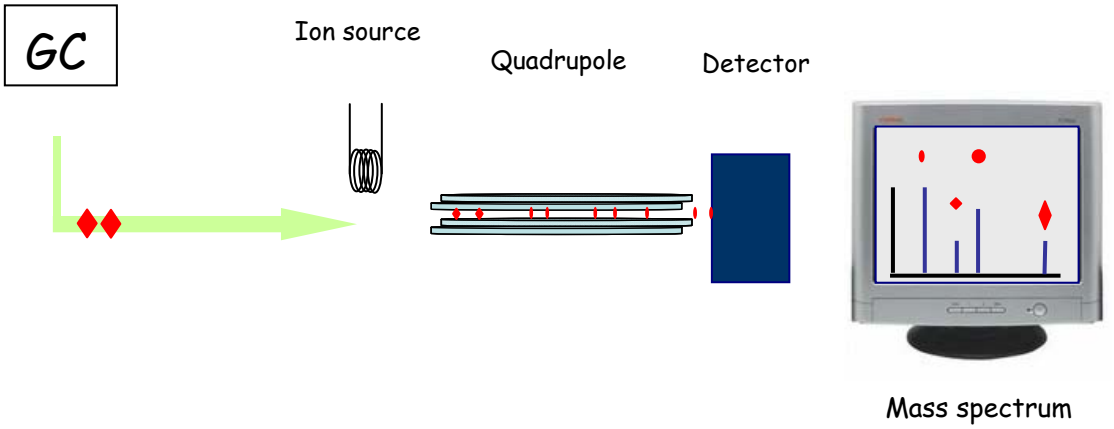


Figure 5. Schematic diagram of a typical gas spectrometer - mass spectrometer system. From the GC the separated analytes are directed through the ion source and ionized to fragment ions and/or molecular ions. The ions produced are separated based on their mass-to-charge (m/z) ratio and detected.

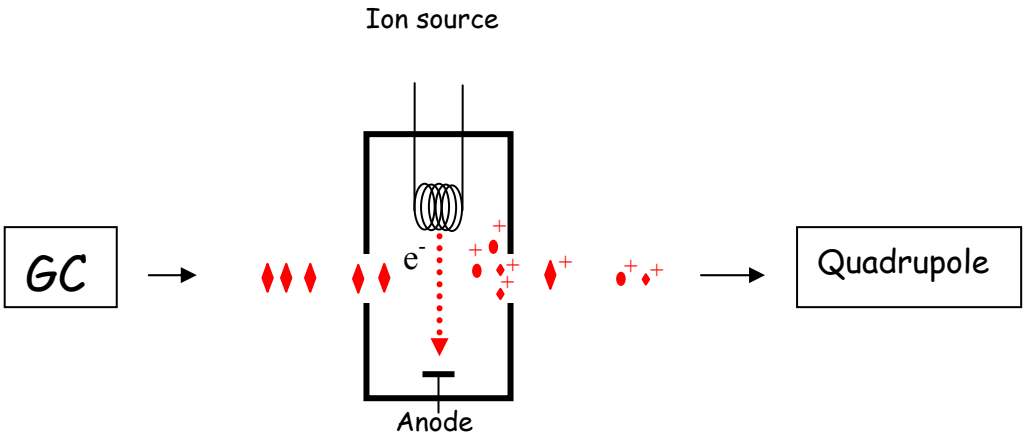


Figure 6. In the ion source the analyte molecules are ionized to charged molecule ions and fragments.

1.5 Validation

A prerequisite for analyzing the potential of exposure to eluted compounds from dental materials is reliable quantification methods, both real time and accelerated measurements. Information about the method validation (Table 3) and results should preferable be included in the reports.

Table 3. Validation parameters for GC-MS quantification methods.

Validation parameters	
Accuracy	The closeness of the measurements to the true value
Limit of detection	The lowest concentration at which the analyte can be detected
Limit of quantification	The lowest concentration at which the analyte can be quantified
Linearity	The correlation between the area of a peak and the amount of the substance
Measuring range	The concentration range within which linearity, precision and accuracy are acceptable
Precision	The agreement between repeated measurements, measured within day and between days
Recovery	The agreements between measurements before and after changed conditions i.e. evaporation and extraction.
Robustness	The method's sensitivity towards small changes in experimental conditions
Selectivity	To what extent an analyte can be determined without interference from other compounds
Sensitivity	The smallest difference in the analyte concentration that can be detected

No dental material is risk-free. Considering that the biological safety of several of the substances leaching is questioned, better knowledge of the level of exposure to the human body is needed. The different products of resin-based dental restorative materials have different potential for causing adverse effects. Thus, in assessment of biocompatibility, both quantitative and qualitative evaluations of the released substances have to be considered. When an allergic reaction to an ingredient in the materials is revealed, knowledge about the composition of the products may give the clinician an opportunity to select an appropriate filling material in clinical dentistry.

2. Aims Of The Study

The general aim of the present work was to characterize resin-based dental restorative materials by analysis of organic substances eluting from the polymerized materials**.**

2.1 Specific Aims

1. To identify organic substances eluting from polymerized resin-based dental restorative materials (Paper I)

2. To establish a method for quantification of low molecular weight organic substances eluting from polymerized resin-based dental restorative materials (Paper II and III)

3. To synthesize tailor-made internal standards for the quantification procedure of monomers in saliva (Paper III)

4. To quantify amouts of eluted substances in saliva (Paper III)

3. Materials and Methods

3.1 Materials

Table 4. Resin-based dental restorative materials investigated in study I-III

Abbreviation		Type of material	Product name	Specifications	Manufacturer
Study I+II	TC	Composite	Tetric Ceram	Color A3, lot B42131	Vivadent Ets. Schaan, Lichtenstein
	FZ	Composite	3M™ Filtek™ Z250	Color A3, lot 19991122	3M Svenska AB, Sollentuna, Sweden
	DY	Compomer	Dyract AP	Color A3, lot 9909000451	Dentsply DeTrey GmbH, Konstanz, Germany
	FU	Resin modified glass ionomer cement	GC Fuji II LC	Color A3, lot 080291	GC Corporation, Tokyo, Japan
Study III	TEC	Composite	Tetric EvoCeram	Color A2, lot H21573	Ivoclar Vivadent AG, FL-9494 Schaan/Liechtenstein
	FZ	Composite	3M ESPE Filtek™ Z250	Color A2, lot 20051213	3M ESPE Dental Products, St.Paul, MN 55144-1000, USA

In study I and II, four materials were included; two composites (FZ and TC), one compomer (DY) and one resin-modified glass ionomer cement (FU) (Table 4). The materials differ in the resin-based matrix ingredients and also in type and weight percent of fillers. Two composites (TEC and FZ) were selected for analysis in study III. FZ in study III differs from FZ in study I and II, by color, factory and lot number.

Table 5. Organic ingredients declared in material safety data sheet (MSDS).

Study I + II				Study III	
TC	**FZ**	**DY**	**FU**	**FZ**	**TEC**
Bis-GMA TEGDMA UEDMA	Bis-EMA Bis-GMA UEDMA TEGDMA CQ	UDMA TCB Resin TMPTMA HMBP CQ DMABEE BHT Alkanoyl-poly-methacrylat	HEMA Polyacrylic acid	Bis-EMA Bis-GMA UEDMA TEGDMA	Bis-GMA UEDMA

3.2 Standards and solvents

All standards used as reference substances were of analytical grade and obtained from Sigma-Aldrich, Oslo, Norway. Diethyl phthalate was purchased from Merck-Schuchardt, Hohenbrunn bei Munchen, Germany, and used as internal standard (I.S.) in study I - III. Ethanol was obtained from Arcus, Bergen, Norway. Methanol, ethyl acetate, NaOH, HCl and the catalyst 10% Pd/charcoal were obtained from Merck, Darmstadt, Germany. Benzenesulfinic acid sodium salt (BSA) was obtained from Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany. All solvents, standards and diethyl phthalate were checked with full scan GC-MS to ensure they contained no compounds interfering with the analysis. $^{1}H_2$ and $^{2}H_2$ gas was purchased from Yara, Norway, and the filter substance Celite 545 was from Kebo lab, Stockholm, Sweden.

Ethyl acetate, ethanol and water was distilled twice and kept in glass bottles, furthermore, analyzed by GC/MS to ensure that they contained no compounds interfering with the analysis. Gloves were not used. All procedures were performed with metal instruments and glassware. In study I and II glassware and instruments were washed twice in double-distilled ethyl acetate, wrapped in aluminum foil and kept at 100 ˚C for at least 12 h before used. The aluminum foil was washed with ethyl acetate before wrapping the equipment. In study III aluminum-wrapped glassware and instruments were heated and kept at 400 ˚C for four hours in a muffle furnace (Carbolite CWF 1200).

3.3 Specimen preparation

Cylindrical stainless steel moulds (study I and II) (Fig. 7) or Teflon moulds (study III) were filled with uncured materials to produce specimens with a diameter of 6 mm and a thickness of 2 mm. Care was taken to avoid air bubbles. The uncured materials were covered with a polyester film (Odus universal-strips, Odus Dental AG) and a glass plate to exclude the oxygen-inhibiting layer, and were polymerized by visible light with an Optilux 400 curing lamp (Demetron Research Corp., Danbury, CT, USA).

The specimens of each material were cured for 40 s in study I and II. Polymerization time of 40 s. was in agreement with specification from the manufacturer for TC, DY and FU. For FZ the manufacturer recommended a polymerization time of 20 s. However, after pilot studies we decided to apply the same curing time for all the materials. The light intensity was measured in study I and II to be above 350 mW/cm^2 by a Curing Radiometer Model 100 (Demetron Research Corp.). In study III the specimens were cured according to the manufacturers; 20 s for both materials. Light intensity was above 500 mW/cm^2, which was in agreement with new recommendation from the producers. The mean weight of the samples in study III was 0.1434 ± 0.0022 grams, (RSD was 3.76%).

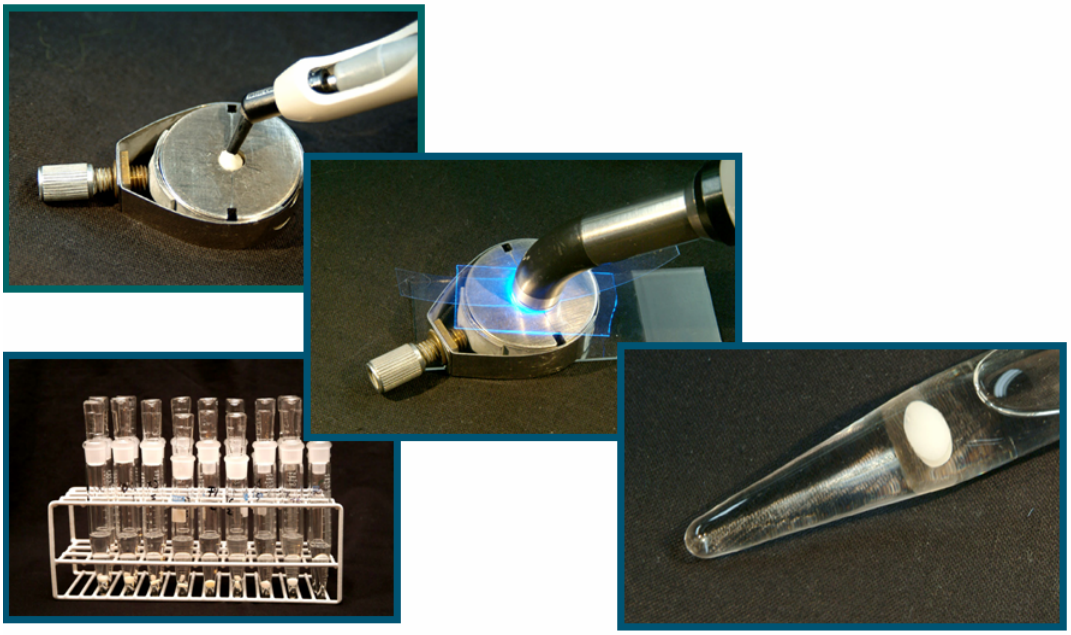


Figure 7. Specimen production and immersion (study I and II).

3.3.1 Immersion

The identity and amounts of eluates from the specimens were studied in three different media. In study I and II two series were produced; one series for immersion in ethanol and one series where Ringer's solution was the immersion media (Fig. 7). In study III the specimens were immersed in saliva; mimicking more realistic

conditions. The saliva was non-stimulated and collected from one individual with no dental restorations. Two hours before the saliva collection tooth brushing and inter-dental hygiene was performed, furthermore from this point on, no intake of food or liquids (except water) and no smoking were allowed until sampling was finished. No intake of alcohol was allowed the day before and until after sampling. After collection the saliva was frozen at -28˚C. Aliquots of the collected saliva was analyzed with GC-MS, and revealed no compounds interfering with the study.

In the study the mass/volume ratio (g/ml) between the specimens and the test solutions were between 1:20 and 1:40 (study I and II) and approximately 1:7 for study III, and the specimens were fully immersed in the test solutions according to ISO 10993-13 (117). Elution in ethanol was carried out for 24 hours, whereas specimens in Ringer's solution were kept eluting for 7 days (study I and II). Immersion time for the specimens in saliva in study III was 24 hours.

3.3.2 Preparation of samples for GC-MS

After completed immersion time the specimens where removed and an internal standard was added to the solution. To obtain higher concentrations of the eluates, the solutions were subject to evaporation and concentration at 50°C. For samples with ethanol as immersion media, the concentrated solutions were transferred to sample vials (Cromacol, London, UK) and injected directly into the GC-MS.

For eluates in saliva and Ringer's solution, transfer of the eluates into an organic solvent was necessary. The leachables were extracted 3 times with 2 ml of distilled ethyl acetate and the extracts pooled for each sample. The pooled extracts were transferred to glass vials, evaporated at 50°C to approximately 200 µl and transferred to sample vials for further analysis in the GC-MS.

3.3.3 Internal standards

In Study I and II diethyl phthalate, 2 μg ml^{-1}, was used as an internal standard. For study III tailor-made internal standards (IS-TD, IS-HD and IS-TH) were synthesized. IS-TD, IS-HD and diethyl phthalate was added to the solution after elution. The internal standard IS-TD was synthesized as follows: TEGDMA (0.5 ml, 1.6 mmol) was dissolved in MeOH (15 ml) and reduced by hydrogenation with $^{2}H_2$ gas on 10% Pd/charcoal at atmospheric pressure for 2 hours. The reaction product was filtered through a short column (Pasteur pipette) filled with Celite 545. For synthesis of IS-HD the same procedure was carried out with HEMA. We used the same method for synthesis of IS-TH but with hydrogenation with $^{1}H_2$ gas instead of $^{2}H_2$ gas. All compounds synthesized were analyzed by GC/MS in full scan mode and mass fragments where chosen for single ion monitoring (SIM) mode suitable for the analysis of the monomers HEMA and TEGDMA.

3.4 Analytical methods

All analysis in study I-III were performed using combined GC-MS. The instrument was a Thermo Quest Trace GC connected to a Finnigan MD 800 quadrupole mass spectrometer. The GC was further equipped with an auto sampler (Finnigan AS-800, Thermo Quest). The instrument and data handling were controlled by the software package Xcalibur (Xcalibur™, Finnigan Corp.). For chromatographic separation we used a capillary column with following specifications: CP-SIL 8 CB wall-coated open tubular (WCOT) low bleed fused silica MS column with column length 30 m, 0.25 mm i.d. and a film thickness of 0.25 μm (Chrompack, Middelburg, The Netherlands). Helium was used as carrier gas with a flow rate of 1 ml min^{-1}, constant flow. Split less injection was used; injector temperature of 250˚C and purge flow of helium gas was 70 ml min^{-1}. The temperature program for the oven: start point at 50˚C, with a rate of 50˚C min^{-1} up to 120˚C, hold time at 120˚C for 5 min, from 120˚C to 230˚C with rate of 10˚C min^{-1}, 230˚C to 280˚C with rate of 120˚C min^{-1} hold time at 280˚C for 1 min.

The syringe was rinsed with ethyl acetate 5 times before and after every injection. A hole with diameter of 3 mm was made in the rubber septum of the sample vial as well as the wash glass, and aluminum foil was used as a seal between the septum and the vial.

Identification of the analytes was performed by using the mass spectrometer in full scan mode (Fig. 8). The mass range for detection was 50-350 m/z. The analytes were identified by comparing the mass spectra with the NIST (National Institute of Science and Technology, Gaithersburg, MD, USA) mass spectra library. Finally, retention time (RT) and mass spectra of the eluates were compared with that of authentic reference substances (Figs. 9-10). For each reference substance and the internal standards, one or two characteristic mass fragments were selected. In study II and III we quantified the compounds by use of integrated peak from these ions in the chromatograms compared with internal standard areas. All integrations were manually adjusted if necessary. Standard curves and response factors were computed. Linearity of area ratios versus quantities was confirmed for all substances. In study III, analyses for quantification were performed in the SIM mode, recording fragments according to Table 6.

Table 6. Base peak ions (most abundant) and qualifying ions for SIM analyses of eluates and internal standards.

Substance	Qualifying ions	Base peaks
TEGDMA	113	69
IS-TH	71	115
IS-TD	73	117
HEMA	87	69
IS-HD	91	73
DEP, Diethyl phthalate	149	177

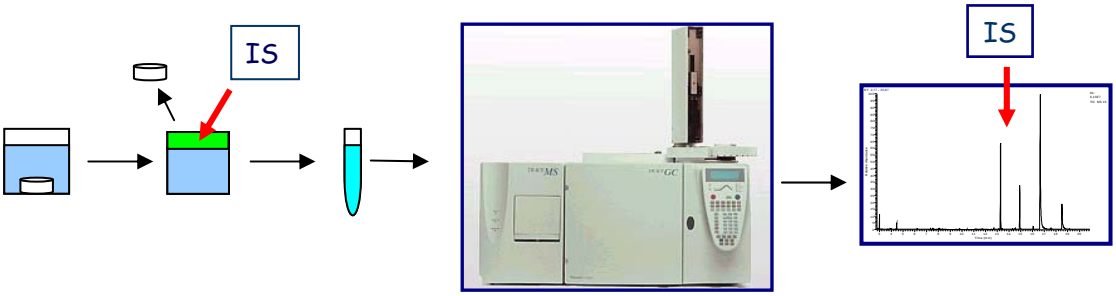


Figure 8. Specimen immersed in ethanol. After 24 hours the specimen was removed and IS added. The sample was concentrated and injected in the GC-MS. Eluted substances were separated and recorded.

Figure 9. The reference substance (TEGDMA) in ethanol, added IS, concentrated and injected in the GC-MS. The recorded chromatogram was compared with the chromatogram from the specimen elution. TEGDMA shows identical retention time with the unknown substance.

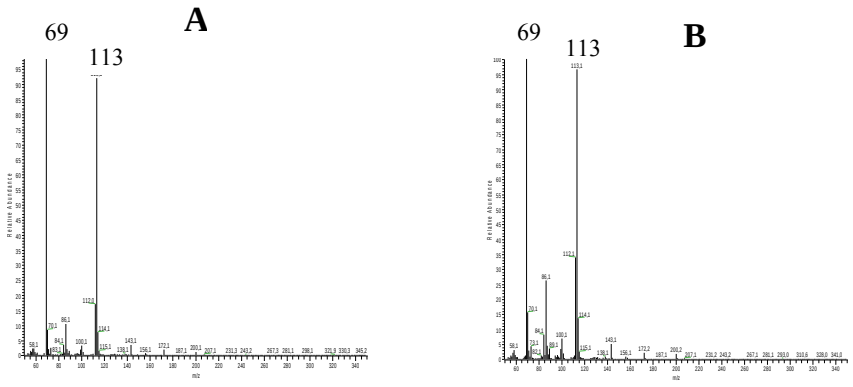


Figure 10. Mass spectra at identical retention times from TEGDMA (A) eluted from a sample and TEGDMA (B) as reference substance. The similarity of mass spectra and ratio between base peak and qualifier ion is shown and confirms the identity of TEGDMA.

3.5 Validation methods

The method for study II and III was validated according to Table 3. In study I parameters regarding quantities were not validated.

Accuracy. In study II: accuracy of measurements was tested in 5 different concentrations; from 1 μg ml^{-1} to 30 μg ml^{-1}. All compounds were tested with 3 parallels. In study III accuracy was tested in 2 concentrations 1 and 5 μg ml^{-1}, three parallels.

Limit of detection – limit of quantification. Lowest limit of detection, LOD is defined as S:N > 3:1 (Signal to Noise), and lowest limit of quantification, LOQ defined as S:N > 10:1, were found by analyzing reference compounds in concentrations from 0.001 to 10 μg ml^{-1} in study II. In study III LOD and LOQ was found by analyzing reference compounds in concentrations from 0.002 to 2 μg ml^{-1}.

Linearity. Ratio of areas of analyte compared to internal standard was plotted against concentrations of the analytes. Calibration curves and response factors were computed with reference substances analyzed with the previously described method in five different concentrations for each compound. In study II these concentrations were from 0.7 μg ml^{-1} to 30 μg ml^{-1} with diethyl phthalate, 2 μg ml^{-1}, as internal standard. In study III area ratios for analyte versus analogue tailor-made standard were computed for HEMA and TEGDMA in concentrations from 0.2 μg ml^{-1} to 10 μg ml^{-1}.

Precision. Precision of measurements was calculated for all analytes in study II in concentrations 1 and 10 μg ml^{-1} and 1 and 5 μg ml^{-1} in study III and given as the standard deviation (S.D.) and relative standard deviation (R.S.D) between repeated measurements for within-day and between-day analysis.

Recovery. Study II: The relative recovery of each analyte compared to the internal standard (I.S.) was tested with a solution of ethyl acetate with reference substances in concentrations 1 and 10 μg ml^{-1} and I.S. of 2 μg ml^{-1}. The ratios of amounts were

compared before and after evaporation. Furthermore, reference substances in concentrations 1 and 10 μg ml^{-1} were added to Ringer's solution and measured after extraction and evaporation. The ratios of signal from reference substances to signal from I.S. were compared to the ratios from the initial solution in ethyl acetate.

Study III: Relative recovery of eluates extracted from saliva compared to eluates extracted from water, was tested in two concentrations 1 and 10 μg ml^{-1}. The relative recovery of HEMA and TEGDMA compared to diethyl phthalate was tested previously with a solution of ethyl acetate with reference substances in concentrations 1 and 10 μg ml^{-1} and diethyl phthalate of 2 μg ml^{-1}. The ratios of signals were compared before and after evaporation, and before and after extraction from water.

Robustness. During evaporation of ethyl acetate to concentrate the sample, it was discovered that considerable losses of HEMA could occur. Systematic experiments were performed to study the effect of evaporation on the loss of HEMA.

Selectivity. The selectivity was ensured by distilling ethyl acetate twice, and redistill ethanol and water to eliminate contaminants. Standards, reference substances and solvents were analyzed with GC-MS in full scan mode to make sure they contained no substances interfering with the analysis.

Sensitivity. Sensitivity is defined as the slope of the linearity, in $y = ax + b$.

3.6 Statistical methods

The quantitative results are presented as μg eluted per unit of surface area of a composite sample (study II; μg mm^{-2}, study III; μg cm^{-2}) and expressed as mean values with associated standard deviations. The Student's Independent-Samples t-test was used to test if observed differences in mean values of each compound eluting from the materials in study II or III were significant. The test was performed in both solutions and between each compound eluting from the various materials. The significance levels were expressed as two-tailed values and a p-value less or equal to

0.05 was considered statistically significant. The calculations were performed by using the software Statistical Package for the Social Science (SPSS) (SPSS Inc., Chicago, USA). For validation the results are presented as mean values with associated standard deviations and percent of standard deviation, %SD or relative percent of standard deviation, %RSD.

3.7 Ethics

Study III is part of a study on leachables from resin based restorative materials in human saliva. This study and an associated establishment of a research bio bank for saliva collected in the study, has been approved by The Regional Committee for Medical Research Ethics, Western Norway.

4. Results

4.1 Identification of organic eluates from four polymer-based dental filling materials (Paper I)

Specimens from two composites, one compomer and one resin-reinforced glass ionomer cement were immersed in ethanol and Ringer's solution. The study demonstrated the diversity in eluted compounds from the four various products analyzed (Fig. 11). Our results indicate the presence of thirty-two eluted substances. Of these 15 were compared with reference substances (Table 7) and each substance had identical full scan mass spectra and retention time with the corresponding reference substance. Reference substances were not commercially available for all substances detected, 17 substances were therefore tentatively identified based on library search, study of the mass spectra and information from literature.

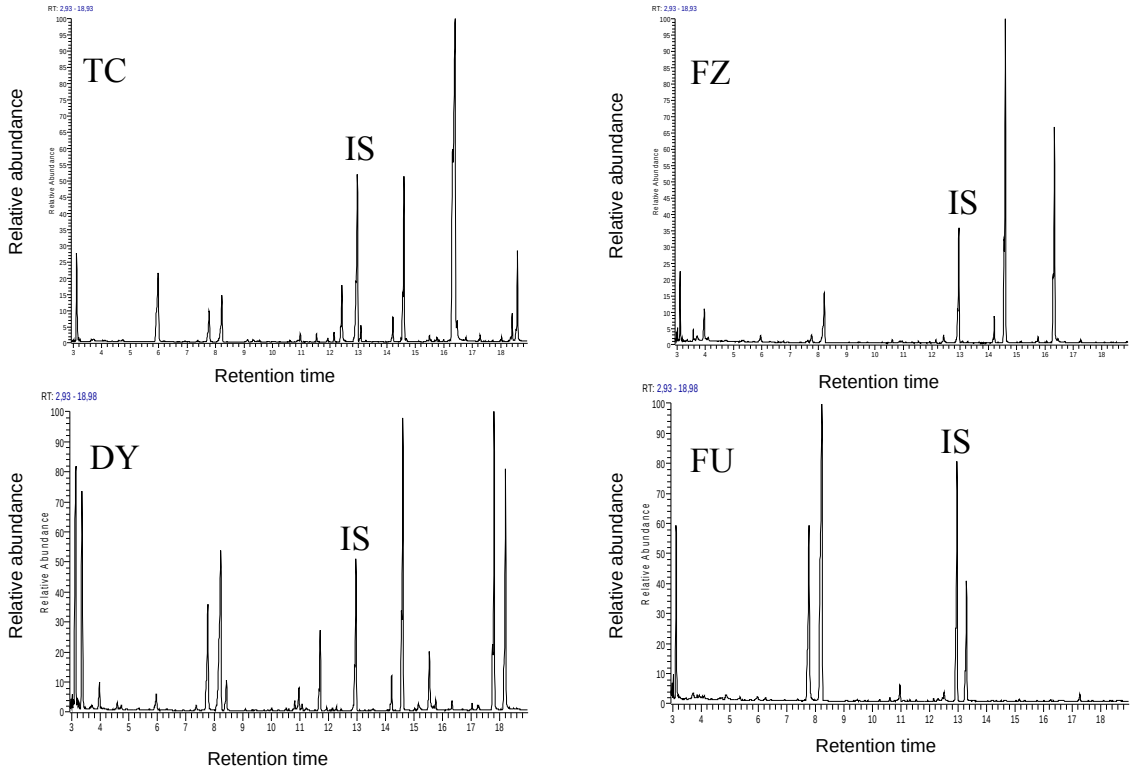
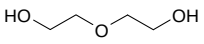
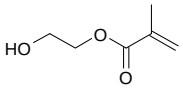
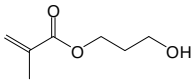
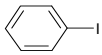
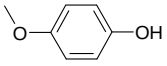
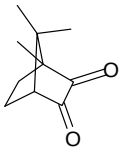
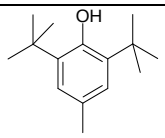
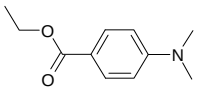
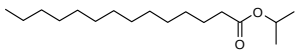
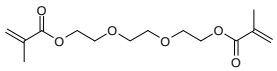
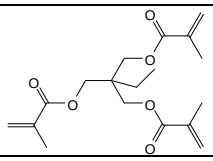
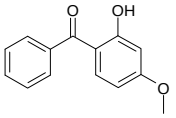
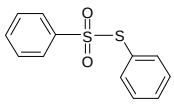
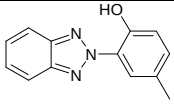
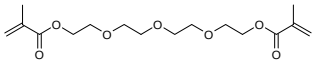


Figure 11. Chromatograms representing elution from TC, FZ, DY and FU in Ringer's solution. Sections from retention times (RT) 2.98 to 18.98 are shown.

Table 7. Eluates with identical mass fragment patterns and retention times to reference substances. Function, CAS numbers, monoisotopic masses, molecular and structural formulas are given.

Eluate	Function	CAS number	Molecular formula	Monoisotopic mass	Structural formula
DEG	Degradation product from monomers	111-46-6	$C_4H_{10}O_3$	106.06	
HEMA	Monomer	868-77-9	$C_6H_{10}O_3$	130.06	
HPMA	Monomer	27813-02-1	$C_7H_{12}O_3$	144.08	
BI	Degradation product from an initiator	591-50-4	C_6H_5I	203.94	
MEHQ	Inhibitor	150-76-5	$C_7H_8O_2$	124.05	
CQ dl-	Initiator	10373-78-1	$C_{10}H_{14}O_2$	166.10	
BHT	Stabilizer	128-37-0	$C_{15}H_{24}O$	220.18	
DMA BEE	Co-initiator	10287-53-3	$C_{11}H_{15}NO_2$	193.11	
IPM		110-27-0	$C_{17}H_{34}O_2$	270.26	
TEGDMA	Monomer	109-16-0	$C_{14}H_{22}O_6$	286.14	
TMPTMA	Monomer	3290-92-4	$C_{18}H_{26}O_6$	338.17	
HMBP	UV-Stabilizer	131-57-7	$C_{14}H_{12}O_3$	228.08	
BTS	Initiator system	1212-08-4	$C_{12}H_{10}O_2S_2$	250.01	
TIN P	UV-Stabilizer	2440-22-4	$C_{13}H_{11}N_3O$	225.09	
TEEGDMA	Monomer	109-17-1	$C_{16}H_{26}O_7$	330.17	

4.2 Quantification of organic eluates from polymerized resin-based dental restorative materials by use of GC/MS (Paper II)

Specimens from four products were immersed in ethanol and Ringer's solution. A difference in numbers, nature and amounts of eluted compounds was observed between materials, Fig. 12.

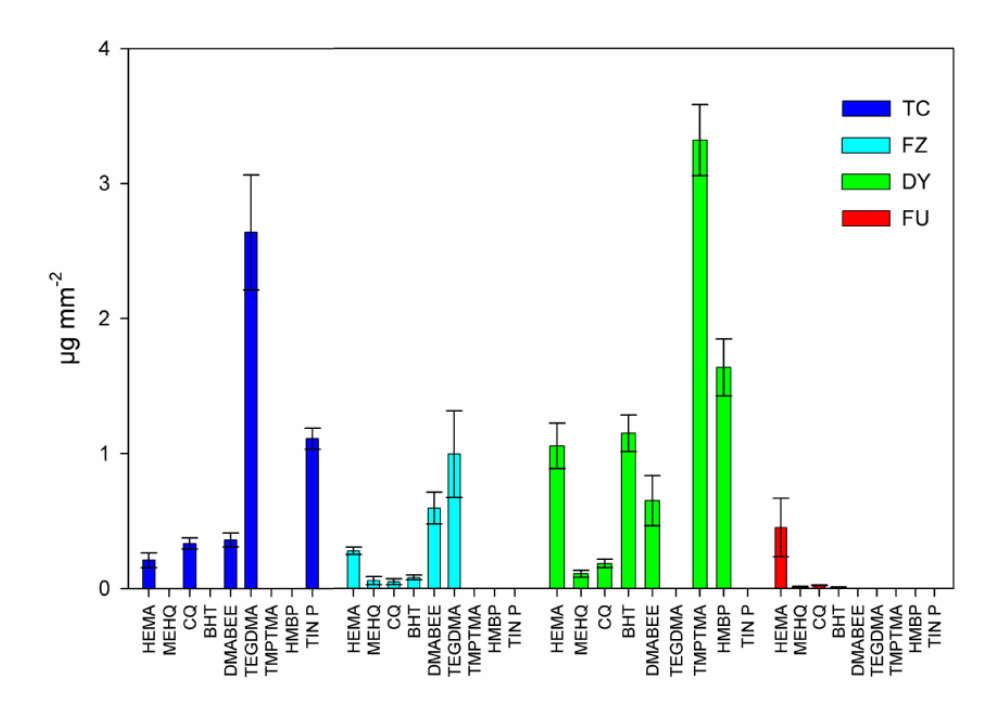


Figure 12. Substances eluted from specimens of TC, FZ, DY and FU in ethanol. The quantities are given in µg mm^{-2} of specimen surface. Mean values (bars) and SDs (vertical lines) are given.

The quantities of eluted compounds from the specimens were up to 20 times higher in ethanol than in Ringer's solution, except for MEHQ from TC. Monomers represented the dominating group of eluted compounds in both solutions. By comparing detected substances and the correlating MSDS from the producers of the four materials, it was shown that MSDS provide limited information about identities of ingredients that are released.

4.3 Quantitative analysis of TEGDMA and HEMA eluted into saliva from two dental composites by use of GC/MS and tailor-made internal standards (Paper III)

This study investigated the elution from two composites immersed in human saliva for 24 hours and the usefulness of tailor-made internal standards for quantification of eluted TEGDMA and HEMA. Three different tailor-made internal standards were synthesized (Figs. 13 and 14). Two of the synthesized standards were applied in the study in addition to diethyl phthalate. Full scan MS spectra showed that the synthesized I.S. contained no traces of the analytes from which they were synthesized. The tailor-made internal standards eluted with retention time close to, but completely separated from their respective analytes. The monomer, HEMA was released from both TEC and FZ , although the observed difference in amount was not statistically significant. TEGDMA eluted from one composite only. The co-initiator DMABEE was found eluted from both materials, but was not quantified in this study.

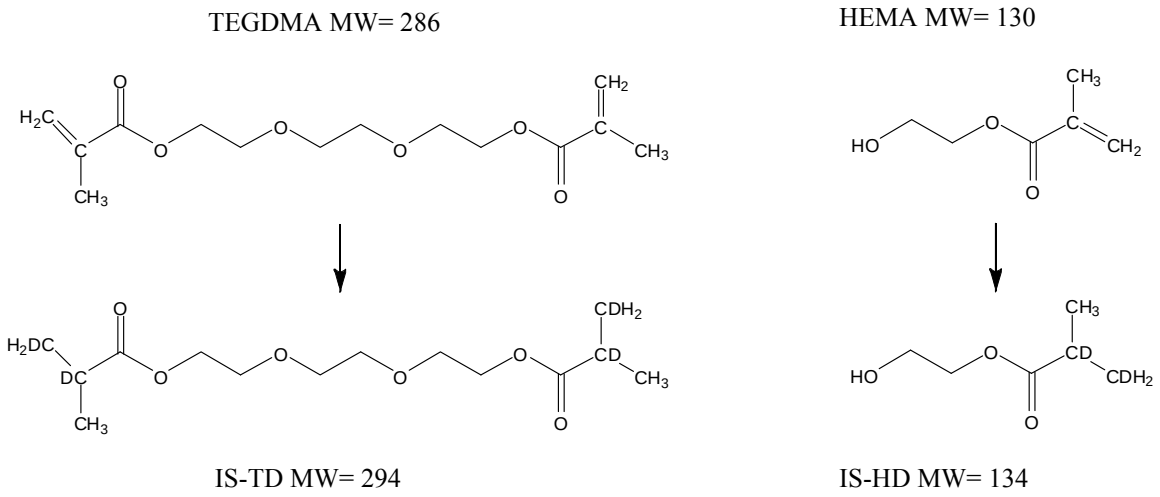


Figure 13. The synthesis of the tailor-made internal standards, IS-TD and IS-HD.

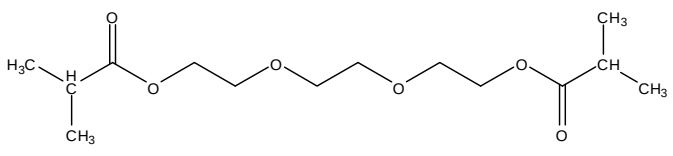


Figure 14. The synthesized internal standard IS-TH.

5. Validation

The quality of measurements is in this thesis expressed through the following validation method and results.

Accuracy. Study II: Average accuracy for measurements of amounts of eluates was 100.75 % with a range from 94 to 108 %. Measurements for BHT showed the highest variation; accuracy was 99.46% for concentrations > 4 µg ml^{-1}, however, in lower concentrations, (between 1 and 4 µg ml^{-1}), accuracy was 122%. In study III average accuracy was 95% with a range from 87 to 100 %, tested in 2 concentrations (1 and 5 µg ml^{-1}).

Limit of detection. The LOD varied between the different substances, and was between 0.01 µg ml^{-1} and 1 µg ml^{-1}. Low molecule-weight compounds needed higher concentrations to be detected in the analysis than the high MW substances (Fig. 15).

Limit of quantification. In study II limit of quantification, LOQ, was between 0.1 µg ml^{-1} and 1 µg ml^{-1}. LOD and LOQ for HEMA and TEGDMA were measured to be in the range of 0.1– 0.5 µg ml^{-1} in study III (Fig. 15).

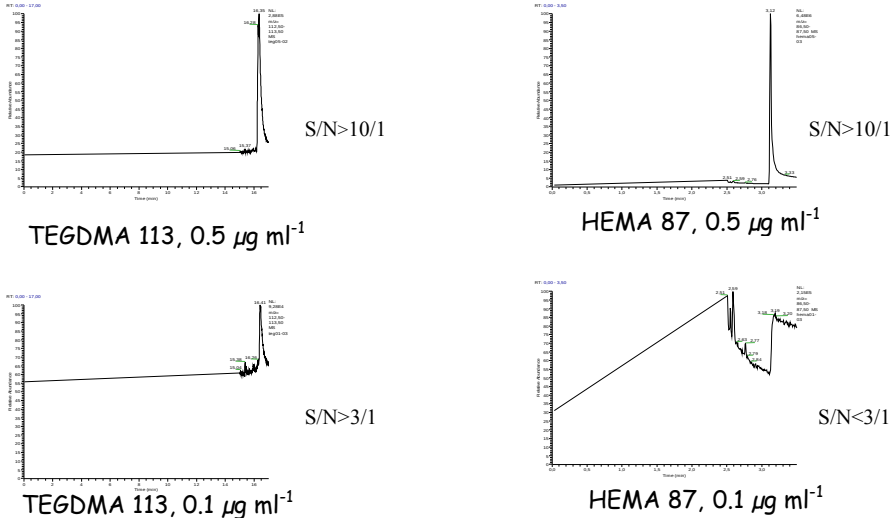


Figure 15. TEGDMA and HEMA analyzed in 0.5 µg ml^{-1} and 0.1 µg ml^{-1} for determination of LOD (S/N>3/1) and LOQ (S/N>10/1) in SIM mode.

Linearity. Study II: Calibration curves and response factors were computed with ratio of areas of analyte compared to internal standard and plotted against concentration of analyte (Fig. 16). The coefficient of determination, R^2, was 0.99 for HEMA, MEHQ, DMABEE, TMPTMA and TIN P, 0.98 for BHT and TEGDMA, 0.94 for HMBP and 0.76 for CQ.

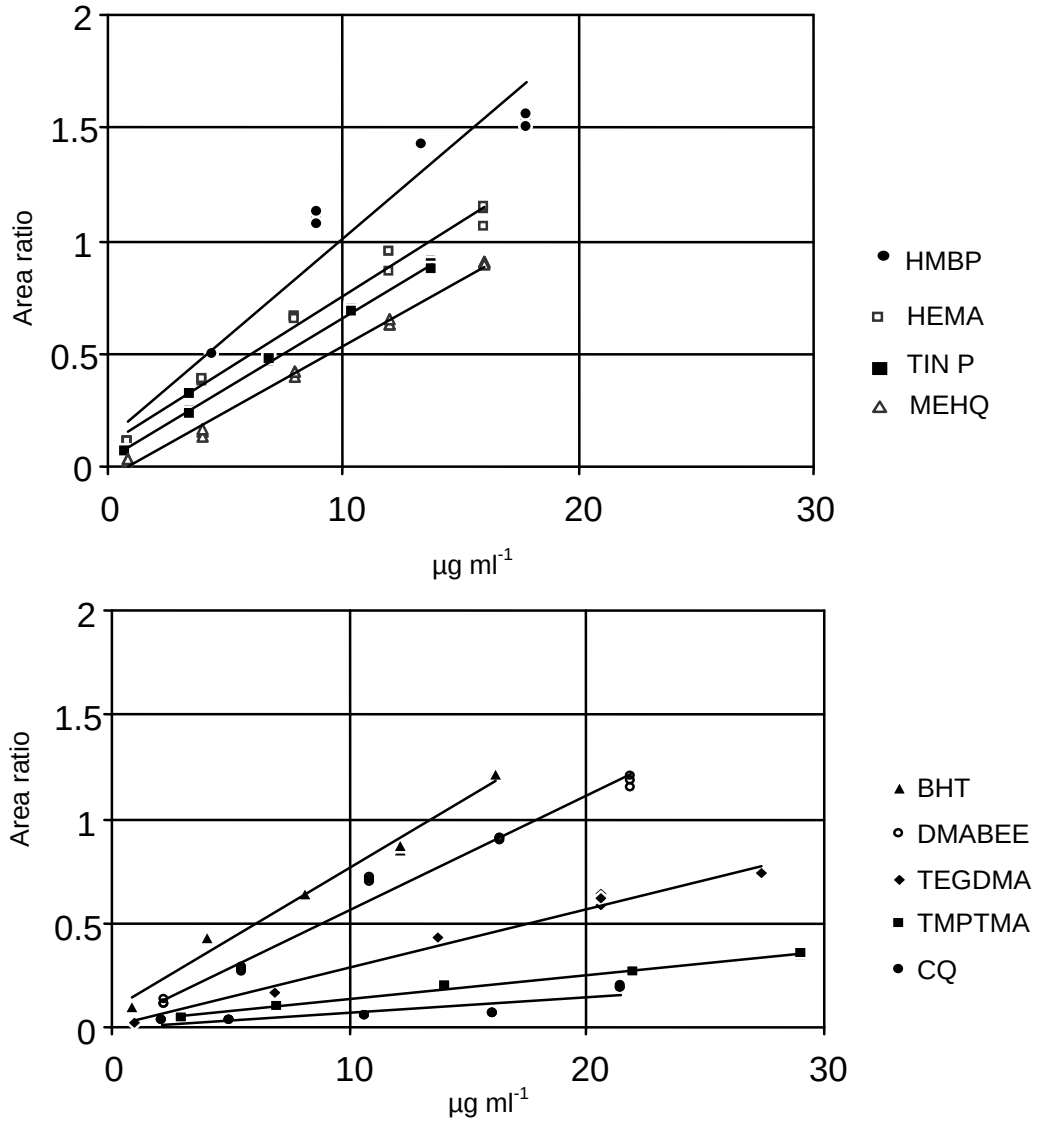


Figure 16. Linearity of all reference compounds. Area ratio of each reference substance / internal standard is plotted against concentration of reference substance.

Study III: For both substances the linearity was good in our selected concentration range (Fig. 17), and the concentrations of eluted substances calculated from the samples were within the linearity range.

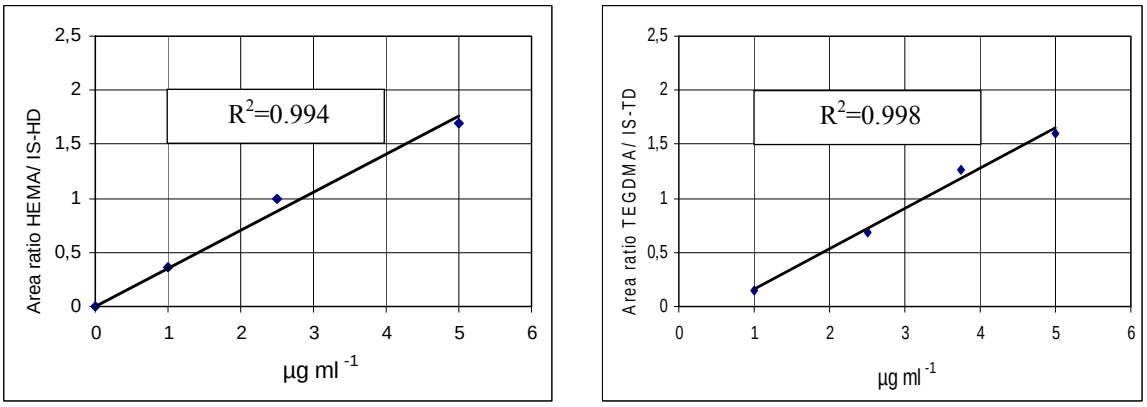


Figure 17. Linearity for HEMA/IS-HD and TEGDMA/IS-TD in study III. R^2 reflects the closeness of measured area ratios to the trend line.

Measuring range. For study II the linearity, precision and accuracy were satisfying between 0.7 µg ml^{-1} to 30 µg ml^{-1} and this was defined as the measuring range. In study III linearity, precision and accuracy were satisfying between 0.2 µg ml^{-1} and 5 µg ml^{-1} for HEMA and 1 µg ml^{-1} and 5 µg ml^{-1} for TEGDMA. These values were defined as the measuring range.

Precision.

For study II within-day precision was between RSD 0.018 and 0.451 % (n=15) for the reference substances, and between-day variation RSD calculated during 2 days, was between 0.019 and 0.512 % (n=10) for all concentrations (Table 8).

Table 8. Precision of the assay in study II. For 10 µg ml -1 concentration of the analytes varied between 9 and 11µg ml-1. For 1 µg ml -1 concentration of the analytes varied between 0.9 and 1.1µg ml-1.

Precision of the assay					
Analyte		Within-day		Between-day	
	Concentration	SD	RSD (%)	SD	RSD (%)
HEMA	10 µg ml^{-1}	0.050	0.451	0.055	0.512
	1 µg ml^{-1}	0.016	0.070	0.011	0.049
MEHQ	10 µg ml^{-1}	0.024	0.236	0.074	0.297
	1 µg ml^{-1}	0.018	0.038	0.012	0.042
CQ	10 µg ml^{-1}	0.003	0.294	0.074	0.369
	1 µg ml^{-1}	0.010	0.043	0.012	0.047
BHT	10 µg ml^{-1}	0.017	0.156	0.021	0.153
	1 µg ml^{-1}	0.002	0.318	0.003	0.335
TEGDMA	10 µg ml^{-1}	0.062	0.079	0.074	0.091
	1 µg ml^{-1}	0.029	0.045	0.033	0.053
TMPTMA	10 µg ml^{-1}	0.150	0.018	0.159	0.019
	1 µg ml^{-1}	0.054	0.029	0.051	0.121
DMABEE	10 µg ml^{-1}	0.009	0.169	0.011	0.196
	1 µg ml^{-1}	0.005	0.138	0.005	0.158
HMBP	10 µg ml^{-1}	0.074	0.202	0.084	0.220
	1 µg ml^{-1}	0.030	0.162	0.030	0.164
TIN P	10 µg ml^{-1}	0.093	0.196	0.107	0.216
	1 µg ml^{-1}	0.045	0.178	0.046	0.180

In study III within-day precision was between RSD 0.03 and 2.5 % (n=10) and between-day variation within two days, was measured as RSD between 0.12 and 1.56 % (n=20).

Relative recovery. The relative recovery of each analyte compared to the I.S. was tested with a solution of ethyl acetate with reference substances in concentrations 1 and 10 µg ml^{-1} with I.S. of 2 µg ml^{-1}. The ratios were compared before and after evaporation. Furthermore, reference substances in concentrations 1 and 10 µg ml^{-1} were added to Ringer's solution and measured after extraction and evaporation. The ratios to I.S. were compared to the ratios from the initial solution in ethyl acetate (Table 9).

Table 9. Relative recovery of the assay. Recovery I: Percent of recovery after evaporation. Recovery II: Percent of recovery after extraction and evaporation. (a: n=5).

Recovery of the assay[a]					
Analyte	Concentration	Recovery I %	SD	Recovery II %	SD
HEMA	10 µg ml^{-1}	75.01	0.12	85.62	0.10
	1 µg ml^{-1}	103.09	0.01	106.66	0.03
MEHQ	10 µg ml^{-1}	93.69	0.06	89.01	0.25
	1 µg ml^{-1}	100.87	0.01	102.28	0.02
CQ	10 µg ml^{-1}	99.16	0.04	90.64	0.01
	1 µg ml^{-1}	90.49	0.01	92.96	0.01
BHT	10 µg ml^{-1}	87.92	0.06	93.58	0.02
	1 µg ml^{-1}	95.90	0.01	94.94	0.02
TEGDMA	10 µg ml^{-1}	94.40	0.05	89.26	0.03
	1 µg ml^{-1}	77.87	0.05	90.81	0.02
TMPTMA	10 µg ml^{-1}	92.20	0.06	86.56	0.05
	1 µg ml^{-1}	84.91	0.04	91.33	0.02
DMABEE	10 µg ml^{-1}	97.67	0.03	96.51	0.01
	1 µg ml^{-1}	94.22	0.03	98.77	0.02
HMBP	10 µg ml^{-1}	90.89	0.08	89.54	0.06
	1 µg ml^{-1}	82.15	0.04	90.88	0.04
TIN P	10 µg ml^{-1}	91.30	0.05	90.14	0.04
	1 µg ml^{-1}	72.18	0.03	87.68	0.02

Study III: Recovery from saliva gave similar results as recovery from water. Relative recovery for HEMA was 98.5 % and for TEGDMA 88 %.

Robustness. We revealed that no loss of HEMA occurred if the reduced volume was 200 µl or higher. Loss occurred when the ethyl acetate phase was reduced completely or close to dryness. With the tailor-made standard the robustness for HEMA was improved. For other substances than HEMA the method was more robust. This was the only step where we found that small changes in the method had major impact in the result.

Selectivity. Internal standards, reference substances and solvents were analyzed with GC-MS in full scan mode and contained no substances interfering with the analysis (Figs. 18 and 19). Analytes and internal standards (DEP, IS- TH and IS-HD) gave

low abundant molecule ions only. For each analyte and each I.S. we used the base peak ion for quantifications in addition to one characteristic ion for each compound as a qualifier ion.

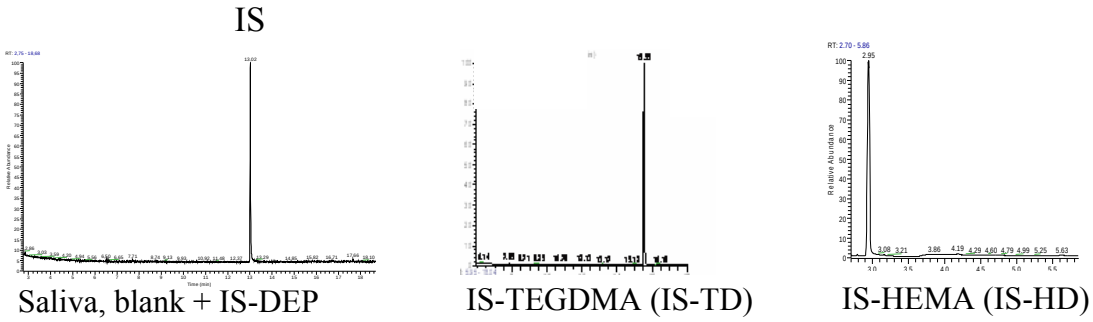


Figure 18. Full scan of saliva blank with IS-DEP, tailor-made IS-TD and IS-HD. In each chromatogram one chromatographic peak representing the internal standard is seen.

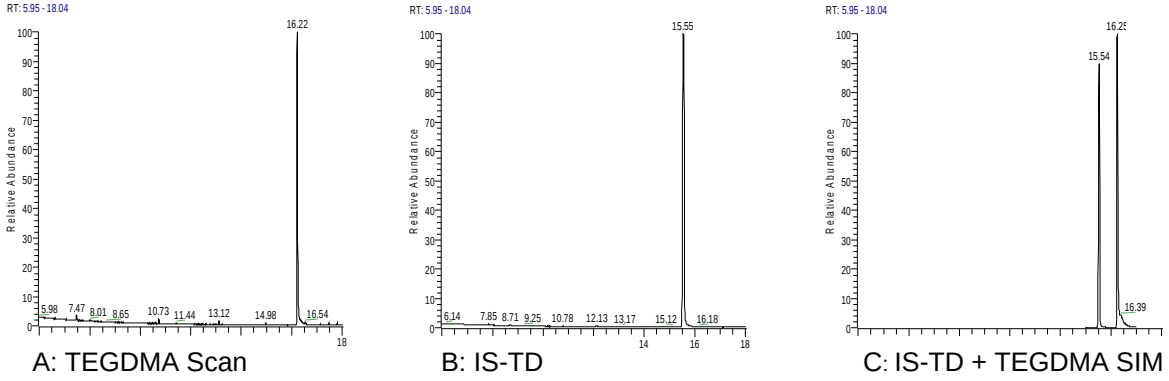


Figure 19. Full scan chromatograms of TEGDMA (A) and IS-TD (B). TEGDMA + IS-TD in SIM mode (C). No contaminating peaks are present.

Sensitivity. For study II the sensitivity was similar to all analytes, except TEGDMA, TMPTMA and CQ expressing lower sensitivity (Fig. 16). In study III SIM mode was applied and the sensitivity was considerably higher for HEMA and TEGDMA (Fig. 17).

6. Discussion

The suitability of a material is determined from a balance between the risks the material poses and the health benefit gained using the material. Exposure to substances released from resin-based materials represents a risk that has to be considered. Investigation of the ingredients by use of Material Safety Data Sheets and the product information does not give a full picture of the ingredients (30), (Paper I and II). In a survey of leachable substances the initial focus should be on the identification of released substances, followed by the determination of the quantities released. In the first section methodological considerations will be addressed, experimental findings and biological considerations discussed in part two.

6.1 Methodological considerations

To emphasize the clinical relevancy in this thesis we have analyzed 6 commercially available resin-based materials. In Paper I and II we chose one product from the compomer group and one among resin-reinforced glass ionomer cements. From the group of composites two materials were chosen due to the considerably higher use of these materials. In study III two different composites were investigated. According to the producer, FZ is a micro hybrid composite, with inorganic filler particles in micro size with scale from 0.01 to 3.5 μm. TEC is characterized by the producer as a nano hybrid composite, containing inorganic filler particles (size 0.040-3μm) in addition to prepolymerized filler particles in nano scale size arranged as clusters. Composition of the materials regarding identity and amounts of ingredients was not known beyond what is declared in the Material Safety Data Sheet from the producers. To further simulate clinical conditions we investigated polymerized specimens since the patients are mainly exposed to cured materials. In pilot studies eluates from uncured materials were analyzed, but these results are not described in this thesis. Uncured materials consisting of unbound monomers represent a situation with a higher potential for adverse effects (118, 119). In recent years focus has been placed on techniques

avoiding contact with the reactive uncured materials. Dental personnel, who are exposed to un-polymerized materials, are recommended to follow a non-touch procedure (120).

In most previous studies characterizing resin-based dental restorative materials, the monomers, which are the main constituents of the polymer network, have been in focus (11, 35, 50, 52, 66, 121-123). Since dental materials contain other substances that may potentially have adverse effects, our aim was to identify (Paper I) a wide range of eluting compounds. Our intention was furthermore to quantify at least one substance from each main group of these compounds (Paper II). Characterization of unknown resin-based substances is difficult because polymers are widely used, and the samples might be contaminated from various sources, including standard laboratory equipment. To avoid such contamination all sources of polymers had to be revealed and controlled. All plastic bottles, pipettes, vials and containers, were replaced with glass ware, and only metal tubing was used. Procedures were performed with solely metal or glass in contact with the specimens, liquids, reference substances and samples. All caps were replaced with glass stoppers or sealed with aluminum foil. Silicon or rubber septa were punched to avoid that the septum particles were transferred into the samples after the needle had perforated the septa. Pilot studies had shown that septum particles eluted compounds that might interfere with the analysis. Evaporation of the samples was prevented with aluminum foil as a seal between the septum and the vial. High selectivity was further ensured by distilling ethyl acetate twice and redistilling ethanol and water. All solvents, blanks and reference compounds were analyzed by GC/MS to reveal any occurrence of compounds interfering with the analysis. If interfering substances occurred redistillation was performed. During specimen production a polyester film was used to cover the uncured materials. The film had been immersed in water and tested for eluting compounds, but released no substances interfering with the analysis. To further

increase the selectivity and lower the limit of quantification and detection, single ion monitoring (SIM) was used in study III.

In study I and II all instruments and glass ware was washed twice with ethyl acetate before use. In study III we avoided this process by heating the glassware and instruments for four hours in a muffle furnace (Carbolite CWF 1200) at 400 ˚C. This procedure was easier and involved no use of solvents.

These procedures ensured that the peaks detected in the chromatograms represented substances from the samples. Two strategies were used for the identification of substances. If a reference substance was available, an identical retention time and an identical mass spectrum in full scan of the reference substance and analyte confirmed the identity. However, not all substances are commercially available, such as degradation products like HC from CQ. Rare compounds may be difficult to obtain, and we were not able to find a supplier of TPSb. For such substances another strategy was used; collecting information from MSDS and comparing with literature, identification by mass spectrum investigation and library search. The mass spectrum shows the ionized fragments produced in the ion source from the original molecule, and a procedure of interpretation of the fragments is useful for identification (124). As a consequence, the identification of compounds not confirmed by reference substances is tentative, judged from the probability. In future studies the use of GC-MS/MS with chemical ionization may improve identification of such substances.

6.1.1 Identification

The identity of chemical compounds can be described in many different ways: by systematic names, generic names, trivial names, trade names and by molecular formulas or structures. The International Union of Pure and Applied Chemistry has developed the IUPAC nomenclature; a system of naming chemical compounds. IUPAC names indicate the specific structure for each compound. However, IUPAC

naming is quite complicated, and compound names may be very long. It was our intention, when characterizing the substances, that the identity of a substance should be described in such a way that the identity is unequivocal. Accordingly, we refer to CAS (Chemical Abstracts Service) registry numbers for chemicals and eluates in study II and III. These numbers are unique numerical identifiers for chemical compounds, polymers, biological sequences, mixtures and alloys, copyrighted by the American Chemical Society. The tailor-made internal standards have to our knowledge not been synthesized before and accordingly have no CAS numbers. In previous studies characterizing resin-based substances, identities are commonly described with trivial names, abbreviations and molecular formulas (31, 32, 36, 125). Structural formulas may accompany the abbreviations of some of the substances (122). By use of CAS numbers, in addition, the identity is unequivocal (123).

To illustrate a confusion regarding identity of chemical compounds, the monomer group urethane dimethacrylates is a relevant example (Table 10). Urethane dimethacrylate is referred to as one compound with several names, or as one name describing several compounds. Methacrylate products are typically mixtures of homologues and isomers (126). A main criterion for identification is match with a reference compound. Since neither CAS numbers nor MW nor structure is usually included in the reports, misinterpretations regarding presence or absence of UEDMA/UDMA are likely to occur. According to MSDS from the producers of TC, FZ, FU and TEC, the ingredient UEDMA has CAS number 72869-86-4. This is the substance we have used as a reference compound. The inconsistent information presented in Table 7, illustrates the importance of using a common term, such as CAS numbers for the identification of substances.

*Table 10. Urethane dimethacrylates with molecular formulas, descriptions, abbreviations, CAS numbers and MW obtained from different sources of information; reports, MSDS, producers and internet databases. # Declared as diurethane dimethacrylate in ChemFinder.com (CambrigdeSoft®, USA). We suggest that it is a spelling error, confirming the confusion. * CAS number is not found in the CAS databases.*

Source of information	Molecular formula	Description	Abbreviation	CAS number	MW
ChemFinder	$C_{23}H_{38}N_2O_8$	Diurethane dimethacrylate	-	41137-60-4	470.56
ChemFinder	$C_{11}H_{15}NO_4$	Urethane dimethacrylate	UEDMA	-	225.24
ChemFinder	-	Diurethane dimethacrylate	-	103597-45-1#	658.87
SciFinder®	$C_{23}H_{38}N_2O_8$	Urethane dimethacrylate	UDMA	72869-86-4	470.56
Örtengren U (127)	$C_{23}H_{38}N_2O_8$	Urethane dimethacrylate	UEDMA	-	470.56
Du MZ, Polymer science (128)	$C_{22}H_{36}N_2O_8$	1,6-bis (methacrylyloxy-2-ethoxycarbonylamino)-2,4,4-trimethylhexane	UDMA	-	-
Sigma-Aldrich, Norway	$C_{23}H_{38}N_2O_8$	Diurethane dimethacrylate	-	72869-86-4	470.56
MSDS Z250	$C_{23}H_{38}N_2O_8$	Diurethane dimethacrylate	-	72869-86-4	470.56
MSDS TEC	$C_{23}H_{38}N_2O_8$	Diurethane dimethacrylate	-	72869-86-4	470.56
MSDS TC	$C_{23}H_{38}N_2O_8$	Diurethane dimethacrylate	-	72869-86-4	470.56
MSDS Fuji II LC	$C_{23}H_{38}N_2O_8$	Diurethane dimethacrylate	-	72869-86-4	470.56
MSDS DY	-	Urethane dimethacrylate	UDMA	10-5883-40-7*	-
Chemotechnique Patch testing	$C_{24}H_{40}N_2O_8$	Urethane dimethacrylate	UEDMA	-	484.6

6.1.2 Immersion media

Assuming that the immersion media is not saturated by eluates, the quantities are not correlated to the volume of the immersion media. The total amount of eluted substance from the specimen is relevant; increasing the amount of media will only dilute the elution solution. Accordingly, using elution per ml as unit therefore gives limited information, but has been used in other studies (24, 35, 66, 129). It is not fully understood how deep the solvent penetrates nor how much and at what rate substances are released from the bulk of the materials. Pelka showed strong correlation between the surface of the specimen and the amount of eluted TEGDMA in a short time elution study (66). In this study the specimen production, size and curing procedure were similar to our study, and elution of TEGDMA was quantified. As TEGDMA was also an abundant and frequent monomer detected in our study, elution correlated to the

surface area of the specimen was chosen as a unit for quantification (μg mm^{-2}). Pilot studies by electron microscopic techniques have shown that the surface of resin-based materials is porous with numerous micro cracks. It is difficult to estimate this micro area; therefore the macro surfaces of the specimens were calculated. This method has also been used in previous studies (52). In the in vivo situation the depth and volume of a restoration may be substantial; frequently the restoration is surrounded by tooth substance and the surface which is exposed to saliva, may be limited. The bulk of the material is mainly exposed to saliva through cracks and diffusion. As a result, for long-term elution the release of compounds from the bulk may be of higher significance for the potential of causing adverse effects. Mohsen et al. found in a cytotoxicity study that chemicals released from the bulk of materials accounted for 60 % of the cellular response (55). From the bulk the eluates may reach the pulp by diffusion through dentin (11).

In a clinical situation the surface is usually contoured and polished, removing the oxygen inhibited layer. We were reluctant to polish the specimens, due to the risk of contaminating the specimens and to increase the temperature, which may potentially increase the degree of conversion, DC. Avoiding the oxygen inhibited layer in vitro may be achieved by polymerization in an oxygen free environment or by covering the specimens with a polyester film and glass plates (18, 66). After scanning electron microscope (SEM) studies of the specimens (250 000 x) we realized that oxygen-free conditions obtained this way may more correctly be described as oxygen-reduced conditions, since oxygen still would be present in numerous micro cracks and pores.

After light-curing specimens were immediately immersed in media, comparable to a clinical situation. At this point polymerization is not completed due to the restricted mobility of compounds in the material, and polymerization will continue for several hours (130). Some studies have allowed drying the specimens for several days until no further weight loss was observed (52). For the quantification of eluates this procedure seems less suitable since polymerization continues after completed light-curing,

accordingly the degree of polymerization before immersion may increase (131). It has been questioned whether the drying process may remove organic leachables as well as water (132). Glass-ionomer cements absorb water in the initial phase and disintegration starts. The initial degradation may be reduced by reinforcing glass ionomer cements with resins. It cannot be excluded that sorption is one factor contributing to the high elution from the compomer (133). Øysaed and Reuter found higher water sorption with a matrix containing more hydrophilic monomers (58). When placing a restoration in the oral cavity, the material will be in contact with the oral tissues and liquids shortly after curing. Thus, immediate immersion in the media was considered more clinically relevant. To further simulate clinical conditions, samples were kept at 37%. Constant agitation was used to simulate saliva interacting with the restoration.

The surrounding environment may influence the chemical stability of the polymer network (36, 123, 134). An in vitro model is a simplification, and the choice of immersion media is of great importance. Therefore, the identity and amounts of eluates from specimens were studied in several media. To estimate a maximum amount of each compound that might be released from the actual filling materials, ethanol was used as an immersion media. Ethanol's ability to enter and swell the polymer matrix in Bis-GMA composites is facilitating the release of residual monomers and other low molecular weight eluates (135). A higher elution in ethanol than water is confirmed in previous studies (9, 136). In addition, ethanol is considered as an immersion media which can mimic the normal degradation that is expected from food and saliva (31).

Immersion in Ringer's solution was used since this solution resembles saliva in pH and salt contents, and has previously been employed as a physiological saline solution for immersion of dental materials (137, 138). In previous studies monomers, but few additives have been detected when water was used as a solvent (32). However, using Ringer's solution we detected a similar pattern of eluates as we did for ethanol,

although in significantly lower concentrations (Paper II). In study III the specimens were immersed in human saliva; mimicking clinical conditions.

6.1.3 Immersion time

Immersion time was chosen according to the nature of the immersion media. Previous studies showing that the process of elution is rapid for most materials and in most types of immersion media. Elution was shown to be practically completed within 24 hours both in water and ethanol (49, 66, 76, 122, 131). In a study analyzing released TEGDMA from composites immersed in water, the highest concentration was observed after 7 days (139). Tanaka et al. reported of continuous elution of TEGDMA for 7 days if cured for 30 sec, although the initial rate was most rapid and the majority of elution completed within the first day (50). Our aim was to detect the majority of potential eluates, therefore 7 days were chosen for the elution in Ringer's solution (Study I and II). Enzymatic degradation of TEGDMA after exposure to salivary enzymes has been demonstrated after 16 days (140). In a serum-containing solution TEGDMA was detected immediately after immersion, however, after 7 days TEGDMA was no longer detectable, due to serum albumin binding (36). Albumin may be present in saliva as a contaminant from either trace of blood or gingival fluid. In solutions without serum, such as saline and water, degradation of TEGDMA was not seen. Thus, to reduce the influence of possible hydrolytic degradation the immersion time was 24 hours for the specimens in saliva in study III.

Hydrophilicity, solubility, porosity, cross-linking and degree of conversion, filler and silane chemistry are important factors when degradation potential is assessed (64). In aqueous solutions the materials are prone to hydrolysis (39); especially the ester linkages in the methacrylate monomers are susceptible to chain scission resulting in shorter segments (63). Previous studies have shown degradation of monomers in water and esterases with formation of formaldehyde, methacrylic acid or Bis-HPPP

over time (66, 67, 121, 141, 142). If enzymes are present, the hydrolysis may be catalyzed (68). Esterification of methacrylates may occur in saliva and accordingly, must be taken into account when analyzing eluates in these media (143). The materials' susceptibility to enzymatic hydrolysis varies with DC and the composition of the materials, TEGDMA being more susceptible than Bis-GMA (140, 143). Studies have also shown that lower pH may increase the hydrolysis of the matrix monomers (68, 123, 145).

6.1.4 Extraction

When Ringer's solution or saliva was used as an immersion media, the analytes were extracted into ethyl acetate since aqueous solvents are not compatible with GC-MS analysis. Pilot studies were performed, and only the first and second extraction contained detectable amounts of substances. Three repeated extractions were sufficient to extract all leachables, in accordance with results from Müller et al. (35). An internal standard was added before the extraction to correct for the potential losses during evaporation.

6.1.5 Evaporation

The reason for evaporating samples was to obtain a higher concentration of eluates. We observed that HEMA was rather volatile and eluted in the GC with RT 3.15 min. (bp is 198 ºC according to supplier Rohm and Hass Company, Philadelphia, USA). Pilot test to account for HEMA during evaporation showed a recovery of 98 % after extraction and evaporation to approximately 200 µl. However, when the samples were evaporated to complete dryness, HEMA was difficult to detect. Care must therefore be taken during the evaporation. This was one reason to tailor-make an internal standard for this substance. Assuming that substances with closely related structures would behave in the same way, an added tailor-made I.S. would correct for the potential losses of HEMA due to evaporation.

It has been claimed that diurethane dimethacrylate, a base monomer with MW 470,6 may disintegrate in the GC (31, 40, 56, 125). According to the producers, all the materials in our study contained this monomer (Table 5). In pilot studies we analyzed samples of ethyl acetate solution with high concentrations of uncured diurethane dimethacrylate (CAS 72869-86-4). Degradation into HEMA, EGDMA and several other fragments was confirmed. In addition, we found BHT which is used as a stabilizer for this monomer. In MSDS the content of BHT was given as 225ppm. The peak of HEMA detected was only slightly higher than the peak of BHT, implicating that only a small percentage of diurethane dimethacrylate disintegrated into HEMA. When the concentration of injected diurethane dimethacrylate was below 1000 µg/ml, we did not detect HEMA or any other fragments. Furthermore, eluted amounts of diurethane dimethacrylate and Bis-GMA are expected to be low, due to the low diffusion coefficient of these substances. Moharamzadeh did not detect UDMA or Bis-GMA in elution from three dental composites in any of 5 aqueous and serum media (36). With the expected low elution of diurethane dimethacrylate, we concluded that using our method the disintegration of diurethane dimethacrylate do not represent any problem for the quantification of HEMA. The dominating eluate from FU was HEMA, which has been detected from FU in several studies (11, 125, 129), whereas Mazzaoui et al. was not able to detect this monomer (122). This was explained by chemical degradation of the monomer. This discrepancy emphasizes the need of standardized analytical methods.

6.1.6 GC versus HPLC

Analysis by use of GC/MS requires compounds that are volatile and stable under typical GC-conditions. Low molecular weight (MW < 350 a.m.u.) eluates are thus well suited for analysis by GC/MS. More polar and high MW monomers i.e. Bis-GMA, Bis-EMA and UEDMA, have low vapor pressure at typical GC-temperatures. It is also suggested that they decompose in the injector, polymerize in the injector port

or interact with the stationary phase in the GC-column, and only decomposition products can be detected (35, 40, 125). For this reason most studies have utilized HPLC in the analysis of larger monomers (11, 24, 49, 52, 66, 129, 136, 146). In some studies HPLC with non-selective detectors have been used, and identification have been based solely on chromatographic retention time (RT) Since different compounds might have the same RT, there might be mistakes with respect to the identity of compounds and systematic errors in the quantitative analysis. To avoid these problems, GC and HPLC can be combined with mass spectrometers giving both a selective and a sensitive detection.

In paper I we identified a large number of different compounds eluted from various dental restorative materials. Some of these findings have previously been described (31, 32, 39, 123). In some cases identification is based on HPLC-UV or HPLC-PB/MS data (32). Our identifications were based on the use of GC-MS, which has several advantages compared to HPLC-UV and HPLC-PB/MS. There is a strong correlation between the structure of a compound and the appearance of its spectrum when using GC-MS and it is easy to reproduce the spectra from laboratory to laboratory, thus extensive mass spectrum libraries are assembled. Use of GC-MS gives access to such libraries and makes it easier to identify unknown compounds detected in a sample. When unknown compounds are present, analyses by GC-MS thus have a great advantage. HPLC-UV gives no or very little structural information, and the separation power of HPLC is inferior compared to GC. HPLC-MS can give some structural information, however the appearance of a spectrum for a given compound can vary from instrument to instrument and only limited numbers of compounds are available in libraries.

Compared to HPLC-UV techniques GC-MS have a better separation power and lower amounts can be detected. Low quantities of released leachables may be of biological relevance, but might be below the detection limit for HPLC-UV methods (18). However, with the recent years developments of new ion sources (Electro spray, ESI

and Atmospheric Pressure Chemical Ionization, APCI), HPLC-MS based methods may represent a selective and sensitive tool for the analysis of eluates from dental restoring materials, especially those with high molecular weight. We observed in pilot studies that the higher molecular weight monomers Bis-GMA, Bis-EMA in addition to diurethane dimethacrylate at high concentrations, were disintegrated with the set injector temperature. Therefore, a complete picture of leachable compounds is not possible to obtain exclusively by GC-MS; however, by combining GC-MS and modern LC-MS methods a full picture may be achieved.

6.1.7 Quantification

The biological effect of several of the substances leaching is questioned, and the fate of the substances eluted into the oral cavity is not fully accounted for. Furthermore, little is known about in which range of elution *in vivo*, biological response or adverse effects may occur. In general toxic effects are dose-dependent (145). This has been observed in studies on apoptosis and necrosis induced by HEMA, TEGDMA (148). Thus, knowledge about the degree of exposure to eluted compounds from the resin-based materials is essential.

In previous studies several analytical methods for quantification of released substances from resin-based dental restorative materials have been applied (31, 32, 35, 50, 52, 66, 122, 123, 125, 149). A wide variation in quantities of eluted compounds has been shown depending on the elution media, immersion time, type of material and degree of cure (32, 34, 36, 52). Studies have suggested that 2-10% of unreacted monomers are elutable (9, 49, 50), however higher elution were found when the materials were inadequately cured (52). In a few studies quantification has been based on the total weight loss of samples after immersion without characterizing specific compounds released (9, 56, 131, 150). In most studies a limited number of substances are quantified, mainly monomers (11, 35, 49, 50, 66, 121-123, 136, 146). The most extensive study characterizing eluates was performed by Spahl et al. (32),

who studied a large number of compounds eluted into water and methanol from four different resin-based materials. In this report, the various eluted compounds were not quantified separately, but were specified as their response in GC/MS or LC/PB/MS compared to the response of caffeine. Lee et al. detected and quantified several monomers and other substances, although using the term "quasi-quantitative" analysis about the quantification of monomers and other substances in his study (31). Similar methods have been applied in the majority of studies on quantification of eluates from resin-based restorative materials. A prerequisite for analyzing the potential of exposure to eluted compounds from dental materials is reliable quantification methods, with both real time and accelerated measurements. To obtain such information, accurate, precise and validated analytical methods are required (Table 3). Methods described in the dental literature do not fulfill or communicate these requirements.

6.1.8 Internal Standards

In quantification studies of eluates from resin-based materials, external standards are most commonly applied (11, 49, 50, 66, 112, 136). The use of an internal standard has several advantages over external standards; correction for loss during sample preparation, corrections for variation in the injection volume and corrections for changes during the chromatographic analysis. A good standard has a RT close to, but well separated from the analytes' RT. Chemical properties should be similar to the analytes' and the I.S. should not be present as a contamination in the sample. The I.S. should also be stable and not undergo reactions with analytes in the sample (151). Internal standards have only been used in a few studies concerning elution from the dental composites (32, 35, 122, 125).

In study I and II we used diethyl phthalate (DEP) as internal standard. DEP has been applied in a previous study (34). The range of retention time of the analytes in study I and II was from 2 to 19 minutes. Diethyl phthalate had RT ~ 13 minutes and eluted

close to a limited number of substances. We decided to improve the method for the quantitative studies of HEMA and TEGDMA, which are substances that have sensitizing properties and has been found responsible for adverse effects *in vitro* (13, 15, 22, 99, 152-154). Our aim was to apply the quantification method for studies of eluates in saliva. Diethyl phthalate is commonly used as a plasticizer and may represent a problem as a contaminant from plastic containers, vials, tubing or others. Contaminations by DEP may thus occur in saliva due to intake of drinks or food or from the dental procedure or saliva sampling. Thus DEP is not an ideal standard for studies of eluates in saliva. Geurtsen and Spahl applied caffeine as an internal standard (32, 39). Caffeine elutes close to TEGDMA, and is therefore useful as an internal standard for quantification of TEGDMA, given that the peaks are well separated. For saliva samples caffeine can not be used as an I.S. because of the high probability of contamination from intake of drinks containing caffeine. This was a major reason to synthesize tailor-made deuterium-containing standards which will definitely not be present in the saliva samples as contaminants (Paper III).

Since HEMA and TEGDMA are common monomers in resin-based dental restorative materials, analogue HEMA and TEGDMA tailor-made internal standards were produced. The synthesis is cheap and simple and theoretically applicable to all methacrylic acids, whether mono-, di-, or tri-functional.

Figure 19 shows that quantitative results for HEMA using DEP as internal standard, resulted in mean values of HEMA slightly lower than when applying the tailor-made internal standard. The result differed significantly for TEGDMA ($p<0.05$). For TEGDMA the corresponding methods show slightly higher values when using DEP. Figure 20 demonstrated that DEP is useful as in internal standard, however, tailor-made standards have the advantage that they will never exist as contaminations in any immersion media used. .

Because of the increase in MW by 4 atomic mass units to the most intense peak ions to both HEMA and TEGDMA, tailor-made standards synthesized with $^{2}H_2$ have

advantages compared to the $^{1}H_2$ analogues. The tailor-made standards elute very close to correlating monomers and are conforming to requirements for internal standards (151).

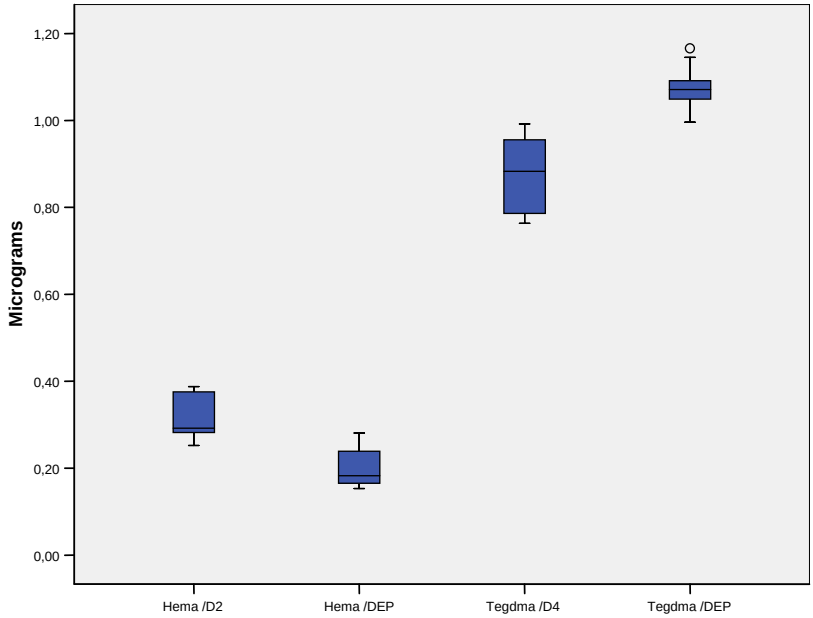


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Figure 20. Quantitative results for HEMA and TEGDMA in study III measured by use of tailor-made and DEP internal standards.

Use of single monitoring mode instead of scan mode increases the sensitivity, and lowers the LOD and LOQ. In Fig. 17 the slope of the trend lines for HEMA and TEGDMA is considerably higher than in Fig. 16, clearly demonstrating the increased sensitivity.

The linearity was good for all substances in our test concentrations. Furthermore, the concentrations of eluted substances were well inside the linearity range, except for TEGDMA from TC and TMPTMA from DY in ethanol which both eluted in slightly higher concentrations. The calibration curve could preferable have be extended up to 50 µg ml^{-1}. Strength of linear relationship or the measure of how well the regression line represents the data between area ratio and concentration is expressed as the

coefficient of determination, R^2. If R^2 is ±1, the correlation is perfect. The low R^2 for CQ is probably due to the low sensitivity, demonstrated in Fig. 17.

6.2 Experimental findings

Our qualitative analysis showed a high diversity of leachable compounds (Paper I), in accordance with other investigations (31, 32, 39). Between materials the elution pattern differs regarding total numbers, types and amounts of single compounds (Paper I, II and III). Thus, the potential for causing adverse effects may vary, however, it is difficult to compare and draw conclusions regarding materials with respect to the biocompatibility.

The highest number of different leachables was found eluting from the compomer (Paper I). This might partly be explained by the complexity of these materials, combining properties of glass ionomer cements with properties of composites. Compomers contain ingredients of the resin system in addition to ingredients from the glass ionomer system. In the quantitative studies the highest amount of a single substance, the monomer TMPTMA, was found in the extracts from the compomer (Paper II). TMPTMA is a tri-functional methacrylate with three sites available for binding to the polymer network. A high quantity of this monomer eluting were not expected, due to the high molecular weight of 338 amu, compared to TEGDMA (MW=286 amu). Larger bulkier molecules are expected to elute at a slower rate than smaller molecules (31). The double bond conversion of TMPTMA has been estimated to 41.7 %, whereas TEGDMA was 68.2 % (155), due to the restricted flexibility of the larger molecule TMPTMA. Considering that three sites are available for binding of TMPTMA, the possibility of TMPTMA and TEGDMA existing as residual monomers is similar or slightly higher for TMPTMA (48).

FU, the resin reinforced glass ionomer cement is also a combination product; mainly a glass ionomer cement with an addition of resin. It is therefore not surprising that from

FU we detected released substances in smaller quantities compared to the other materials investigated (Paper I and II). From this point of view FU may be considered as more biocompatible, however, biocompatibility is a complex issue and the elution of resin-based components is one of several parameters to consider. The mechanical properties of resin reinforced glass ionomer cements are known to be inferior compared to composites. This is the reason why these materials cannot be placed in stress-bearing restorations, and the clinical indications are limited compared to composites.

TEGDMA is not a given ingredient in the material TEC, and was not found eluted from this material in study III. From a biological point of view this material may represent an advantage due to the absence of TEGDMA. Reducing or eliminating the content of TEGDMA has long been an aim for producers because of potentially unwanted biological effects (22, 125, 152, 156). In other respects these two materials are similar regarding the elution of low molecular organic substances (Paper III), which are regarded as most potent concerning adverse effects (73). TEGDMA has been quantified eluted in distilled water from composites (35). The results indicated that the co-monomer's composition had an impact on leachability; UEDMA samples produced less eluted TEGDMA than Bis-GMA-based samples. TEGDMA-containing materials in our study all contained both UDMA and Bis-GMA.

Decomposition products from two initiator system, in addition to the camphoroquinone system, were found (Paper I and II). From FZ we detected BI, which might be a degradation product from DPICl, known as an initiator (125). From FU butylphenyl sulfone (BPS) was detected. This is probably a degradation product from the initiator benzene sulfinic acid sodium salt. In the MSDS this compound is given with CAS number 168-71-7. In our analysis the compound detected was CAS number 873-55-2. This misspelling in the MSDS has been confirmed by the producer of FU. In addition, CQ is given as ingredients in both materials. Our findings showed that several initiators may be used in the same material. It further demonstrated that

the information from the producers is not complete (Paper II). In cases when an allergy is questioned, a full record of ingredients is needed, since minor amounts of substances may be sufficient for an allergic reaction to occur. DPICl and TEGDMA were suggested to be the primary cause for cytotoxic reactions, thus leaching should be minimized (125).

Quantities of HEMA and TEGDMA leaching in saliva were considerable lower than quantities eluting in Ringer's solution (Paper II and III). This is not easy to explain. In pilot studies we immersed specimens in 1 ml, 2 ml and 5 ml saliva to investigate if saturation of eluates occurred in low volumes of saliva. Saturation was not demonstrated. Saliva contains, in contrast to Ringer's solution, enzymes that are able to degrade the polymer network (141, 157). Degradation of TEGDMA to TEGMA after 16 days has been reported, when composites were exposed to cholesterol esterase, an enzyme found in human saliva (140). The immersion time for investigation of elution in saliva was 24 hours. If degradation to TEGMA occurred in significant amounts, a peak should appear in the chromatogram B1 shown in Paper III, Fig. 3. Since a reference substance for TEGMA was not available, all peaks in the chromatogram were analyzed. None of the observed peaks have mass fragments correlating to TEGMA. Our conclusion is that TEGMA is not present in quantities above the detection limit after 24 hours. Thus, it seems unlikely that this degradation process is responsible for the small quantities of detected TEGDMA.

The material FZ in study I and II differ from FZ in study III concerning production year, place, color and batch number. The chemical composition may have been changed, resulting in a lower elution. This might be revealed by testing the elution from FZ from study III in Ringer's solution. Further experiments are needed to investigate these observations.

6.3 Biological considerations

The process of degradation of resin-based dental restorative materials and the biological implications are still not fully revealed. Adverse effects *in vitro* has been reported for most substances identified in study I (Table 2), however, the correlation to the *in vivo* situation is difficult to asses. Only when the concentrations at which tissue is exposed to, are known, the implications can be considered with a degree of certainty. Considering the extensive use of resin-based materials in dentistry, the frequency of adverse reactions has been regarded as low (156, 159). Several reports conclude that resin-based materials are safe, due to the low concentrations eluted (33, 89). However, a continuous characterization of dental materials is required for safety evaluation. (89). Allergic reactions are considered as dose-independent. Information about ingredients present in quantities less than 1 % weight are not requested for MSDS (113). Thus, characterization of dental materials is important to reveal ingredients responsible for adverse reactions in patients and dental personnel. When allergic contact dermatitis or delayed hypersensitivity to a dental allergen is suspected the patient may undergo a diagnostic patch testing to identify a potential allergen. A standardized epicutan patch testing series was proposed in 1983 by Axéll et al. (160). Modern epicutan tests for dental materials, such as the Dental Screening DS-1000, are based on this series. Epicutan tests consist of a battery of different substances, and are available from several producers. A positive test result is not necessarily equivalent with an allergy (147). When the patient's history and symptoms correlates with an allergic reaction to this substance, care has to be taken to avoid the allergen. The results from patch tests for some substances may be questioned, Table 8.

*Table 11. Producer's information about substances tested in DS-1000 dental series compared to substances declared and/or detected in dental materials in our study. Information from the database SCI-finder is shown. * not specified.*

Source of information	Molecular formula	Specified name	Abbreviation	CAS number	Monoisotopic mass
Chemotechnique Patch test DS-1000	$C_{24}H_{40}N_2O_8$	Urethane dimethacrylate	UEDMA	*	484,6
SCI finder®	$C_{23}H_{38}N_2O_8$	Urethane dimethacrylate	UDMA	72869-86-4	470.56
Ingredients in FZ, TC, TEC, FU	$C_{23}H_{38}N_2O_8$	Diurethane dimethacrylate	*	72869-86-4	470.56
Chemotechnique Patch test DS-1000	$C_7H_8O_2$	Methyl hydroquinone	MHQ	95-71-6	124.05
Eluted from FZ, TC, TEC, FU	$C_7H_8O_2$	Hydroquinone monomethyl ether	MEHQ	150-76-5	124.05

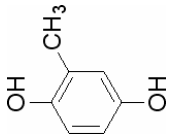

Figure 21. Methyl hydroquinone (CAS 95-71-6)

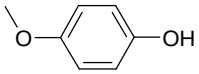

Figure 22. Hydroquinone monomethyl ether (CAS 150-76-5)

In the Dental Screening test DS-1000, methyl hydroquinone MHQ (CAS 95-71-6) is given as an inhibitor used in composites (Fig. 21). According to a personal communication with producers of resin-based materials methyl hydroquinone is not commonly used in composites at present. However, the inhibitor monomethyl ether hydroquinone, MEHQ (CAS 150-76-5) is extensively used (Fig. 22), also in the raw materials. MEHQ was detected in study I and II, in accordance with findings in other reports (123, 161). A cross reaction between MHQ and MEHQ might be a possible

explanation for using MHQ, however, we have not found reports to confirm this cross reaction. UEDMA is included in the patch series. This monomer is described in many forms (Table 10). In patch test DS-1000 UEDMA has a CAS number and a molecular weight different from the ingredient in the composite materials in our study. In addition, we have not seen this substance detected in other studies. It might be questioned if a positive reaction to UEDMA or MEHQ in the patch test is relevant to ingredients present in composite materials currently available. A consequence of a positive reaction in the patch test to one of these substances may be an advice to avoid this compound. For dental personnel the result might be inability to work, thus, clarification of this matter is important.

7. CONCLUSIONS

From the findings of the thesis the following conclusion can be drawn:

The applied GC-MS based method is well suited for qualitative and quantitative analysis of low molecular weight (MW< 350 amu) organic leachables from dental restorative materials, and can be used to characterize and compare such materials with respect to the number and the quantitative amounts of leachables.

Extreme care must be taken to avoid contaminating compounds.

The inconsistency in use of names and abbreviations implies that CAS numbers should accompany names when substances are characterized. Use of CAS numbers ensures an unequivocal identity of substances.

Use of the tailor-made internal standards improved the accuracy and the precision for quantitative elution studies of the monomers HEMA and TEGDMA. The method is useful as one of the tools for testing biocompatibility of resin-based dental materials.

Substances representing several groups of organic ingredients from the polymer matrix are elutable, such as monomers, stabilizers, inhibitors, initiators, co-initiators and production enhancers.

The findings clearly demonstrate that the materials do contain several ingredients not given in the MSDS.

Each material has its specific elution pattern, regarding numbers, nature and amounts of eluates. The biocompatibility, including the sensitizing potential of the materials may therefore vary.

Monomers represented, separately and as a group, the highest amount of eluted substances.

8. FUTURE PERSPECTIVES

To investigate the elution from resin-based dental restorative materials *in vivo*. The method will be applied for analysis of saliva from patients who has received treatment with resin-based dental restorative materials. Both identification and quantification of the eluted compounds will be performed;

by use of GC/MS to characterize low weight organic substances

by use of LC/MS to characterize low weight organic and water-soluble substances and larger monomers

To synthesize tailor-made internal standards for the quantification of other monomers

To apply the method to evaluate the biocompatibility of non-dental biomaterials, i.e. orthopedic materials

To develop and apply methods for the analysis of other monomers, such as Bis-GMA, Bis-EMA and UEDMA by use of modern LC- MS techniques.

Our synthesis method may be used to produce tailor-made internal standards for other methacrylates.

9. REFERENCES

1. Bowen R. Properties of a silica-reinforced polymer for dental restorations. J Am Dent Assoc 1963;66:57-63.
2. Sundberg H, Mejàre I, Espelid I, Tveit AB. Swedish dentists' decisions on preparation techniques and restorative materials. Acta Odontol Scand. 2000;58 (3):135-41.
3. Tveit AB, Espelid I, Skodje F. Restorative treatment decisions on approximal caries in Norway. Int Dent J. 1999;49 (3):165-72.
4. Forss H, Widström E. From amalgam to composite: selection of restorative materials and restoration longevity in Finland. Acta Odontol Scand. 2001;59 (2):57-62.
5. Espelid I, Tveit AB, Mejàre I, Sundberg H, Hallonsten AL. Restorative treatment decisions on occlusal caries in Scandinavia. Acta Odontol Scand. 2001;59 (1):21-7.
6. Mjör IA, Moorhead JE, Dahl JE. Selection of restorative materials in permanent teeth in general dental practice. Acta Odontol Scand. 1999;57 (5):257-62.
7. Norwegian Board of Health. The use of dental filling materials in Norway; 1999.
8. Directorate for Health and Social Affairs. A National Clinical Guideline for the Use of Dental Filling Materials : Information for dental health care personnel. Oslo, Norway: Directorate for Health and Social Affairs, Norway; 2003.
9. Ferracane JL. Elution of leachable components from composites. J Oral Rehabil. 1994;21 (4):441-52.
10. Olea N, Pulgar R, Perez P, Olea-Serrano F, Rivas A, Novillo-Fertrell A, et al. Estrogenicity of resin-based composites and sealants used in dentistry. Environ Health Perspect. 1996 Mar;104 (3):298-305.
11. Hamid A, Okamoto A, Iwaku M, Hume WR. Component release from light-activated glass ionomer and compomer cements. J Oral Rehabil. 1998;25 (2):94-9.
12. Bouillaguet S, Wataha JC, Hanks CT, Ciucchi B, Holz J. In vitro cytotoxicity and dentin permeability of HEMA. J Endod. 1996 May;22 (5):244-8.
13. Geurtsen W, Lehmann F, Spahl W, Leyhausen G. Cytotoxicity of 35 dental resin composite monomers/additives in permanent 3T3 and three human primary fibroblast cultures. J Biomed Mater Res. 1998;41 (3):474-80.
14. Bouillaguet S, Wataha JC, Hanks CT, Ciucchi B, Holz J. In vitro cytotoxicity and dentin permeability of HEMA. J Endod. 1996;22 (5):244-8.
15. Hanks CT, Strawn SE, Wataha JC, Craig RG. Cytotoxic effects of resin components on cultured mammalian fibroblasts. J Dent Res. 1991;70 (11):1450-5.
16. Atsumi T, Murata J, Kamiyanagi I, Fujisawa S, Ueha T. Cytotoxicity of photosensitizers camphorquinone and 9-fluorenone with visible light irradiation on a human submandibular-duct cell line in vitro. Arch Oral Biol. 1998;43 (1):73-81.
17. Becher R, Kopperud HM, Al RH, Samuelsen JT, Morisbak E, Dahlman HJ, et al. Pattern of cell death after in vitro exposure to GDMA, TEGDMA, HEMA and two compomer extracts. Dent Mater. 2006 Jul;22 (7):630-40.
18. Wataha JC, Rueggeberg FA, Lapp CA, Lewis JB, Lockwood PE, Ergle JW, et al. In vitro cytotoxicity of resin-containing restorative materials after aging in artificial saliva. Clin Oral Investig. 1999;3 (3):144-9.
19. Heil J, Reifferscheid G, Waldmann P, Leyhausen G, Geurtsen W. Genotoxicity of dental materials. Mutat Res. 1996;368 (3-4):181-94.

20. Schweikl H, Schmalz G, Spruss T. The induction of micronuclei in vitro by unpolymerized resin monomers. J Dent Res. 2001 Jul;80 (7):1615-20.

21. Schweikl H, Schmalz G. Triethylene glycol dimethacrylate induces large deletions in the hprt gene of V79 cells. Mutat Res. 1999;438 (1):71-8.

22. Schweikl H, Altmannberger I, Hanser N, Hiller KA, Bolay C, Brockhoff G, et al. The effect of triethylene glycol dimethacrylate on the cell cycle of mammalian cells. Biomaterials. 2005 Jul;26 (19):4111-8.

23. Schafer TE, Lapp CA, Hanes CM, Lewis JB, Wataha JC, Schuster GS. Estrogenicity of bisphenol A and bisphenol A dimethacrylate in vitro. J Biomed Mater Res. 1999;45 (3):192-7.

24. Wada H, Tarumi H, Imazato S, Narimatsu M, Ebisu S. In vitro estrogenicity of resin composites. J Dent Res. 2004 Mar;83 (3):222-6.

25. Al-Hiyasat AS, Darmani H, Elbetieha AM. Leached components from dental composites and their effects on fertility of female mice. Eur J Oral Sci. 2004 Jun;112 (3):267-72.

26. Geukens S, Goossens A. Occupational contact allergy to (meth)acrylates. Contact Dermatitis. 2001;44 (3):153-9.

27. Örtengren U, Andreasson H, Karlsson S, Meding B, Barregård L. Prevalence of self-reported hand eczema and skin symptoms associated with dental materials among Swedish dentists. Eur J Oral Sci. 1999;107 (6):496-505.

28. Hensten-Pettersen A. Skin and mucosal reactions associated with dental materials. Eur J Oral Sci. 1998;106 (2 Pt 2):707-12.

29. Kanerva L, Lahtinen A, Toikkanen J, Forss H, Estlander T, Susitaival P, et al. Increase in occupational skin diseases of dental personnel. Contact Dermatitis. 1999 Feb;40 (2):104-8.

30. Kanerva L, Henriks-Eckerman ML, Jolanki R, Estlander T. Plastics/acrylics: material safety data sheets need to be improved. Clin Dermatol. 1997;15 (4):533-46.

31. Lee SY, Huang HM, Lin CY, Shih YH. Leached components from dental composites in oral simulating fluids and the resultant composite strengths. J Oral Rehabil. 1998;25 (8):575-88.

32. Spahl W, Budzikiewicz H, Geurtsen W. Determination of leachable components from four commercial dental composites by gas and liquid chromatography/mass spectrometry. J Dent. 1998;26 (2):137-45.

33. Arenholt-Bindslev D, Breinholt V, Preiss A, Schmalz G. Time-related bisphenol-A content and estrogenic activity in saliva samples collected in relation to placement of fissure sealants. Clin Oral Investig. 1999 Sep;3 (3):120-5.

34. Lygre H, Høl PJ, Solheim E, Moe G. Organic leachables from polymer-based dental filling materials. Eur J Oral Sci. 1999;107 (5):378-83.

35. Müller H, Olsson S, Söderholm K-J. The effect of comonomer composition, silane heating, and filler type on aqueous TEGDMA leachability in model resin composites. Eur J Oral Sci. 1997 Aug;105 (4):362-8.

36. Moharamzadeh K, Van Noort R, Brook IM, Scutt AM. HPLC analysis of components released from dental composites with different resin compositions using different extraction media. J Mater Sci Mater Med. 2007 Jan;18 (1):133-7.

37. Polydorou O, Trittler R, Hellwig E, Kummerer K. Elution of monomers from two conventional dental composite materials. Dent Mater. 2007 Apr 2.

38. Rogalewicz R, Batko K, Voelkel A. Identification of organic extractables from commercial resin-modified glass-ionomers using HPLC-MS. J Environ Monit. 2006 Jul;8 (7):750-8.

39. Geurtsen W. Substances released from dental resin composites and glass ionomer cements. Eur J Oral Sci. 1998;106 (2 Pt 2):687-95.

40. Spahl W, Budzikiewicz, H. Qualitative analysis of dental resin composites by gas and liquid chromatography/mass spectrometry. Fresenius J Anal Chem. 1994;350:684-91.

41. Anusavice K. Phillips' Science of Dental Materials. Eleventh Edition ed: Saunders; 2003.

42. Bouillaguet S, Shaw L, Gonzalez L, Wataha JC, Krejci I. Long-term cytotoxicity of resin-based dental restorative materials. J Oral Rehabil. 2002;29 (1):7-13.

43. Roulet JF. Degradation of Dental Polymers. Basel, S: Karger; 1987.

44. Ferracane JL. Materials in Dentistry, Principles and Applications. Second edition ed. Philadelpia, USA: Lippincott Williams & Wilkins; 2001.

45. Imazato S, McCabe JF, Tarumi H, Ehara A, Ebisu S. Degree of conversion of composites measured by DTA and FTIR. Dent Mater. 2001;17 (2):178-83.

46. Asmussen E. Factors affecting the quantity of remaining double bonds in restorative resin polymers. Scand J Dent Res. 1982;90 (6):490-6.

47. Jancar J, Wang W, DiBenedetto AT. On the heterogeneous structure of thermally cured bis-GMA/TEGDMA resins. J Mater Sci Mater Med. 2000 Nov;11 (11):675-82.

48. Bland MH, Peppas NA. Photopolymerized multifunctional (meth)acrylates as model polymers for dental applications. Biomaterials. 1996 Jun;17 (11):1109-14.

49. Inoue K, Hayashi I. Residual monomer (Bis-GMA) of composite resins. J Oral Rehabil. 1982 Nov;9 (6):493-7.

50. Tanaka K, Taira M, Shintani H, Wakasa K, Yamaki M. Residual monomers (TEGDMA and Bis-GMA) of a set visible-light-cured dental composite resin when immersed in water. J Oral Rehabil. 1991;18 (4):353-62.

51. Rueggeberg FA, Craig RG. Correlation of parameters used to estimate monomer conversion in a light-cured composite. J Dent Res. 1988;67 (6):932-7.

52. Munksgaard EC, Peutzfeldt A, Asmussen E. Elution of TEGDMA and BisGMA from a resin and a resin composite cured with halogen or plasma light. Eur J Oral Sci. 2000;108 (4):341-5.

53. Caughman WF, Caughman GB, Shiflett RA, Rueggeberg F, Schuster GS. Correlation of cytotoxicity, filler loading and curing time of dental composites. Biomaterials. 1991;12 (8):737-40.

54. Munksgaard EC, Asmussen E, Peutzfeldt A. Plastfyldingsmaterialer. København: Odontologisk Boghandels forlag; 1995.

55. Mohsen NM, Craig RG, Hanks CT. Cytotoxicity of urethane dimethacrylate composites before and after aging and leaching. J Biomed Mater Res. 1998;39 (2):252-60.

56. Rathbun MA, Craig RG, Hanks CT, Filisko FE. Cytotoxicity of a BIS-GMA dental composite before and after leaching in organic solvents. J Biomed Mater Res. 1991;25 (4):443-57.

57. Ruyter E. [Composite dental restorative materials]. Tandlaegebladet. 1985;89 (2):67-70.

58. Øysæd H, Ruyter IE. Water sorption and filler characteristics of composites for use in posterior teeth. J Dent Res. 1986;65 (11):1315-8.

59. Söderholm KJ, Yang MC, Garcea I. Filler particle leachability of experimental dental composites. Eur J Oral Sci. 2000;108 (6):555-60.

60. Willems G, Lambrechts P, Braem M, Celis JP, Vanherle G. A classification of dental composites according to their morphological and mechanical characteristics. Dent Mater. 1992 Sep;8 (5):310-9.

61. Mitra SB, Wu D, Holmes BN. An application of nanotechnology in advanced dental materials. J Am Dent Assoc. 2003 Oct;134 (10):1382-90.

62. Söderholm KJ. Relationship between compressive yield strength and filler fractions of PMMA composites. Acta Odontol Scand. 1982;40 (3):145-50.

63. Santerre JP, Shajii L, Leung BW. Relation of dental composite formulations to their degradation and the release of hydrolyzed polymeric-resin-derived products. Crit Rev Oral Biol Med. 2001;12 (2):136-51.

64. Ferracane JL. Hygroscopic and hydrolytic effects in dental polymer networks. Dent Mater. 2006 Mar;22 (3):211-22.

65. Bruck SD. Leachable components in polymeric medical implants and disposables: a brief critical review. Med Prog Technol. 1990;16 (3):131-43.

66. Pelka M, Distler W, Petschelt A. Elution parameters and HPLC-detection of single components from resin composite. Clin Oral Investig. 1999 Dec;3 (4):194-200.

67. Øysæd H, Ruyter IE, Sjøvik Kleven IJ. Release of formaldehyde from dental composites. J Dent Res. 1988;67 (10):1289-94.

68. Göpferich A. Mechanisms of polymer degradation and erosion. Biomaterials. 1996 Jan;17 (2):103-14.

69. Williams DF. Definitions in biomaterials. New York: Elsevier Science Publ.; 1987.

70. Schmalz G. Concepts in biocompatibility testing of dental restorative materials. Clin Oral Investig. 1997;1 (4):154-62.

71. ISO standards. ISO-10993-16. Biological evaluation of medical devices. Part 16. International Organization for Standardization. 1997.

72. Schweikl H, Spagnuolo G, Schmalz G. Genetic and cellular toxicology of dental resin monomers. J Dent Res. 2006 Oct;85 (10):870-7.

73. Jacobsen N, Hensten Pettersen A. Materiallære, Biological Aspects of Dental Materials. Oslo, Norway: University of Oslo, Department of Odontology; 2000.

74. Kanerva L, Rantanen T, Aalto-Korte K, Estlander T, Hannuksela M, Harvima RJ, et al. A multicenter study of patch test reactions with dental screening series. Am J Contact Dermat. 2001 Jun;12 (2):83-7.

75. Wataha JC, Hanks CT, Strawn SE, Fat JC. Cytotoxicity of components of resins and other dental restorative materials. J Oral Rehabil. 1994;21 (4):453-62.

76. Hume WR, Gerzia TM. Bioavailability of components of resin-based materials which are applied to teeth. Crit Rev Oral Biol Med. 1996;7 (2):172-9.

77. Noda M, Wataha JC, Kaga M, Lockwood PE, Volkmann KR, Sano H. Components of dentinal adhesives modulate heat shock protein 72 expression in heat-stressed THP-1 human monocytes at sublethal concentrations. J Dent Res. 2002 Apr;81 (4):265-9.

78. Schweikl H, Schmalz G, Rackebrandt K. The mutagenic activity of unpolymerized resin monomers in Salmonella typhimurium and V79 cells. Mutat Res. 1998;415 (1-2):119-30.

79. Pagoria D, Lee A, Geurtsen W. The effect of camphorquinone (CQ) and CQ-related photosensitizers on the generation of reactive oxygen species and the production of oxidative DNA damage. Biomaterials. 2005 Jul;26 (19):4091-9.

80. Al-Hiyasat AS, Darmani H, Elbetieha AM. Effects of resin based dental composites on fertility of male mice. Eur J Oral Sci. 2002;110 (1):44-7.

81. Imai Y. Comments on "Estrogenicity of resin-based composites and sealants used in dentistry". Environ Health Perspect. 1999 Jun;107 (6):A290-2.

82. Fung EY, Ewoldsen NO, St Germain HA, Jr., Marx DB, Miaw CL, Siew C, et al. Pharmacokinetics of bisphenol A released from a dental sealant. J Am Dent Assoc. 2000 Jan;131 (1):51-8.

83. Schmalz G, Preiss A, Arenholt-Bindslev D. Bisphenol-A content of resin monomers and related degradation products. Clin Oral Investig. 1999 Sep;3 (3):114-9.

84. Pulgar R, Olea-Serrano MF, Novillo-Fertrell A, Rivas A, Pazos P, Pedraza V, et al. Determination of bisphenol A and related aromatic compounds released from bis-GMA-based composites and sealants by high performance liquid chromatography. Environ Health Perspect. 2000;108 (1):21-7.

85. Noda M, Komatsu H, Sano H. HPLC analysis of dental resin composites components. J Biomed Mater Res. 1999;47 (3):374-8.

86. Lewis JB, Rueggeberg FA, Lapp CA, Ergle JW, Schuster GS. Identification and characterization of estrogen-like components in commercial resin-based dental restorative materials. Clin Oral Investig. 1999;3 (3):107-13.

87. Nathanson D, Lertpitayakun P, Lamkin MS, Edalatpour M, Chou LL. In vitro elution of leachable components from dental sealants. J Am Dent Assoc. 1997;128 (11):1517-23.

88. Kadoma Y, Tanaka M. Acid and base-catalyzed hydrolysis of bisphenol A-related compounds. Dent Mater J. 2000 Jun;19 (2):139-52.

89. Söderholm KJ, Mariotti A. BIS-GMA--based resins in dentistry: are they safe? J Am Dent Assoc. 1999;130 (2):201-9.

90. Manabe A, Kaneko S, Numazawa S, Itoh K, Inoue M, Hisamitsu H, et al. Detection of bisphenol-A in dental materials by gas chromatography-mass spectrometry. Dent Mater J. 2000 Mar;19 (1):75-86.

91. Hashimoto Y, Moriguchi Y, Oshima H, Nishikawa J, Nishihara T, Nakamura M. Estrogenic activity of chemicals for dental and similar use in vitro. J Mater Sci Mater Med. 2000 Aug;11 (8):465-8.

92. Munksgaard EC. Permeability of protective gloves by HEMA and TEGDMA in the presence of solvents. Acta Odontol Scand. 2000 Apr;58 (2):57-62.

93. Piirila P, Hodgson U, Estlander T, Keskinen H, Saalo A, Voutilainen R, et al. Occupational respiratory hypersensitivity in dental personnel. Int Arch Occup Environ Health. 2002 Apr;75 (4):209-16.

94. Björkner B. Sensitizing capacity of ultraviolet curable acrylic compounds. Lund; 1984.

95. Lüders G. Exogen induzierte erkrankungen der mundschleimhaut. Z Hautkr 1986;62:603-12.

96. Kanerva L. Cross-reactions of multifunctional methacrylates and acrylates. Acta Odontol Scand. 2001;59 (5):320-9.

97. Kaga M, Noda M, Ferracane JL, Nakamura W, Oguchi H, Sano H. The in vitro cytotoxicity of eluates from dentin bonding resins and their effect on tyrosine phosphorylation of L929 cells. Dent Mater. 2001 Jul;17 (4):333-9.

98. Clemmensen S. Sensitizing potential of 2-hydroxyethylmethacrylate. Contact Dermatitis. 1985;12 (4):203-8.

99. Kanerva L, Estlander T, Jolanki R. Allergic contact dermatitis from dental composite resins due to aromatic epoxy acrylates and aliphatic acrylates. Contact Dermatitis. 1989;20 (3):201-11.

100. Wallenhammar LM, Örtengren U, Andreasson H, Barregård L, Björkner B, Karlsson S, et al. Contact allergy and hand eczema in Swedish dentists. Contact Dermatitis. 2000;43 (4):192-9.

101. Schnuch A, Geier J, Uter W, Frosch PJ. Patch testing with preservatives, antimicrobials and industrial biocides. Results from a multicentre study. Br J Dermatol. 1998 Mar;138 (3):467-76.

102. Yoshii E. Cytotoxic effects of acrylates and methacrylates: relationships of monomer structures and cytotoxicity. J Biomed Mater Res. 1997;37 (4):517-24.

103. Fujisawa S, Kadoma Y, Komoda Y. 1H and 13C NMR studies of the interaction of eugenol, phenol, and triethyleneglycol dimethacrylate with phospholipid liposomes as a model system for odontoblast membranes. J Dent Res. 1988;67 (11):1438-41.

104. Terakado M, Yamazaki M, Tsujimoto Y, Kawashima T, Nagashima K, Ogawa J, et al. Lipid peroxidation as a possible cause of benzoyl peroxide toxicity in rabbit dental pulp--a microsomal lipid peroxidation in vitro. J Dent Res. 1984;63 (6):901-5.

105. Issa Y, Watts DC, Brunton PA, Waters CM, Duxbury AJ. Resin composite monomers alter MTT and LDH activity of human gingival fibroblasts in vitro. Dent Mater. 2004 Jan;20 (1):12-20.

106. Szczurko C, Dompmartin A, Michel M, Moreau A, Leroy D. Photocontact allergy to oxybenzone: ten years of experience. Photodermatol Photoimmunol Photomed. 1994;10 (4):144-7.

107. Collins P, Ferguson J. Photoallergic contact dermatitis to oxybenzone. Br J Dermatol.1994;131(1):124-9

108. Niklasson B, Björkner B. Contact allergy to the UV-absorber Tinuvin P in plastics. Contact Dermatitis. 1989;21 (5):330-4.

109. Björkner B, Niklasson B. Contact allergy to the UV absorber Tinuvin P in a dental restorative material. Am J Contact Dermat. 1997;8 (1):6-7.

110. Ratanasathien S, Wataha JC, Hanks CT, Dennison JB. Cytotoxic interactive effects of dentin bonding components on mouse fibroblasts. J Dent Res. 1995;74 (9):1602-6.

111. Schwengberg S, Bohlen H, Kleinsasser N, Kehe K, Seiss M, Walther UI, et al. In vitro embryotoxicity assessment with dental restorative materials. J Dent. 2005 Jan;33 (1):49-55.

112. The Council of the European Communities. Council Directive 93/42/EEC of 14 June 1993 Concerning Medical Devices. OJ No L 169/1, 1993.

113. The Norwegian Labour Inspection Authority. Compilation and distribution of safety data sheets for dangerous chemicals The Norwegian Labour Inspection Authority; 2003.

114. ISO-4049:2000. Dentistry -- Polymer-based filling, restorative and luting materials. International organization for standardization 2000.

115. ISO 10993-1:1992. Biological evaluation of medical devices - Part 1: Evaluation and testing. International organization for standardization 1993.

116. Dempster A. A new Method of Positive Ray Analysis. Phys Rev 1918;11:381-5.

117. ISO 10993-13:1998. Biological evaluation of medical devices -- Part 13: Identification and quantification of degradation products from polymeric medical devices. International organization for standardization 1998.

118. Kaga M, Noda M, Ferracane JL, Nakamura W, Oguchi H, Sano H. The in vitro cytotoxicity of eluates from dentin bonding resins and their effect on tyrosine phosphorylation of L929 cells. Dent Mater. 2001;17 (4):333-9.

119. Rueggeberg FA, Dlugokinski M, Ergle JW. Minimizing patients' exposure to uncured components in a dental sealant. J Am Dent Assoc. 1999;130 (12):1751-7.

120. Schmalz G. The biocompatibility of non-amalgam dental filling materials. Eur J Oral Sci. 1998;106 (2 Pt 2):696-706.

121. Santerre JP, Shajii L, Tsang H. Biodegradation of commercial dental composites by cholesterol esterase. J Dent Res. 1999;78 (8):1459-68.

122. Mazzaoui SA, Burrow MF, Tyas MJ, Rooney FR, Capon RJ. Long-term quantification of the release of monomers from dental resin composites and a resin-modified glass ionomer cement. J Biomed Mater Res. 2002;63 (3):299-305.

123. Örtengren U, Langer S, Göransson A, Lundgren T. Influence of pH and time on organic substance release from a model dental composite: a fluorescence spectrophotometry and gas chromatography/mass spectrometry analysis. Eur J Oral Sci. 2004 Dec;112 (6):530-7.

124. McLafferty FW. Interpretation of Mass Spectra. Mill Valley, California; 1980.

125. Geurtsen W, Spahl W, Leyhausen G. Residual monomer/additive release and variability in cytotoxicity of light-curing glass-ionomer cements and compomers. J Dent Res. 1998;77 (12):2012-9.

126. Schedle A, Ivanova M, Kenndler E. Determination of ethoxylated bisphenol A dimethacrylate monomers in dental composites by micellar electrokinetic chromatography. J Chromatogr A. 2003 Mar 21;990 (1-2):231-7.

127. Örtengren U. On composite resin materials. Degradation, erosion and possible adverse effects in dentists. Swedish dental journal. 2000 (141):1-61.

128. Du MZ, Y. Modification of Silica Nanoparticles and Their Application in UDMA Dental Polymeric Composites. Polymer Composites. 2007;28:198-207.

129. Kawai K, Takaoka T. Fluoride, hydrogen ion and HEMA release from light-cured GIC restoratives. Am J Dent. 2002 Jun;15 (3):149-52.

130. Watts DC, Amer OM, Combe EC. Surface hardness development in light-cured composites. Dent Mater. 1987 Oct;3 (5):265-9.

131. Ferracane JL, Condon JR. Rate of elution of leachable components from composite. Dent Mater. 1990;6 (4):282-7.

132. Øilo G. Biodegradation of dental composites/glass-ionomer cements. Adv Dent Res. 1992;6:50-4.

133. Um CM, Øilo G. The effect of early water contact on glass-ionomer cements. Quintessence Int. 1992 Mar;23 (3):209-14.

134. Yap AU, Tan SH, Wee SS, Lee CW, Lim EL, Zeng KY. Chemical degradation of composite restoratives. J Oral Rehabil. 2001;28 (11):1015-21.

135. Asmussen E. Softening of BISGMA-based polymers by ethanol and by organic acids of plaque. Scand J Dent Res. 1984;92 (3):257-61.

136. Kim JG, Chung CM. Elution from light-cured dental composites: comparison of trimethacrylate and dimethacrylate as base monomers. J Biomed Mater Res B Appl Biomater. 2005 Feb 15;72 (2):328-33.

137. Takahashi Y, Hasegawa J, Kameyama Y. Dissolution of metallic mercury in artificial saliva and eleven other solutions. Dent Mater. 1989;5 (4):256-9.

138. Lygre H, Solheim E, Gjerdet NR. Leaching from denture base materials in vitro. Acta Odontol Scand. 1995;53 (2):75-80.

139. Örtengren U, Wellendorf H, Karlsson S, Ruyter IE. Water sorption and solubility of dental composites and identification of monomers released in an aqueous environment. J Oral Rehabil. 2001 Dec;28 (12):1106-15.

140. Finer Y, Santerre JP. Influence of silanated filler content on the biodegradation of bisGMA/TEGDMA dental composite resins. J Biomed Mater Res A. 2007 Apr;81 (1):75-84.

141. Munksgaard EC, Freund M. Enzymatic hydrolysis of (di)methacrylates and their polymers. Scand J Dent Res. 1990 Jun;98 (3):261-7.

142. Shajii L, Santerre JP. Effect of filler content on the profile of released biodegradation products in micro-filled bis-GMA/TEGDMA dental composite resins. Biomaterials. 1999;20 (20):1897-908.

143. Finer Y, Santerre JP. Salivary esterase activity and its association with the biodegradation of dental composites. J Dent Res. 2004 Jan;83 (1):22-6.

144. Finer Y, Santerre JP. Biodegradation of a dental composite by esterases: dependence on enzyme concentration and specificity. J Biomater Sci Polym Ed. 2003;14 (8):837-49.

145. Örtengren U, Andersson F, Elgh U, Terselius B, Karlsson S. Influence of pH and storage time on the sorption and solubility behaviour of three composite resin materials. J Dent. 2001;29 (1):35-41.

146. Gerzina TM, Hume WR. Diffusion of monomers from bonding resin-resin composite combinations through dentine in vitro. J Dent. 1996 Jan-Mar;24 (1-2):125-8.

147. Vamnes JS, Lygre GB, Grönningsæter AG, Gjerdet NR. Four years of clinical experience with an adverse reaction unit for dental biomaterials. Community Dent Oral Epidemiol. 2004 Apr;32 (2):150-7.

148. Samuelsen JT, Dahl JE, Karlsson S, Morisbak E, Becher R. Apoptosis induced by the monomers HEMA and TEGDMA involves formation of ROS and differential activation of the MAP-kinases p38, JNK and ERK. Dent Mater. 2007 Jan;23 (1):34-9.

149. Yap AU, Lee HK, Sabapathy R. Release of methacrylic acid from dental composites. Dent Mater. 2000;16 (3):172-9.

150. Hofmann N, Renner J, Hugo B, Klaiber B. Elution of leachable components from resin composites after plasma arc vs standard or soft-start halogen light irradiation. J Dent. 2002;30 (5-6):223-32.

151. Grob RL. Modern Practice of Gas Chromatography. Third Edition ed. New York: John Wiley & Sons, Inc.; 1995.

152. Stanislawski L, Lefeuvre M, Bourd K, Soheili-Majd E, Goldberg M, Perianin A. TEGDMA-induced toxicity in human fibroblasts is associated with early and drastic glutathione depletion with subsequent production of oxygen reactive species. J Biomed Mater Res A. 2003 Sep 1;66 (3):476-82.

153. Lefeuvre M, Amjaad W, Goldberg M, Stanislawski L. TEGDMA induces mitochondrial damage and oxidative stress in human gingival fibroblasts. Biomaterials. 2005 Sep;26 (25):5130-7.

154. Noda M, Wataha JC, Lewis JB, Kaga M, Lockwood PE, Messer RL, et al. Dental adhesive compounds alter glutathione levels but not glutathione redox balance in human THP-1 monocytic cells. J Biomed Mater Res B Appl Biomater. 2005 May;73 (2):308-14.

155. Cowperthwaite GF, Foy JJ, Malloy MA. The nature of the crosslinking matrix found in dental composite filling materials and sealants. New York: Plenum Press; 1981.

156. Darmani H, Al-Hiyasat AS. The resin monomer triethylene glycol dimethacrylate exhibits reproductive toxicity in male mice. Reprod Fertil Dev. 2005;17 (4):401-6.

157. Freund M, Munksgaard EC. Enzymatic degradation of BISGMA/TEGDMA-polymers causing decreased microhardness and greater wear in vitro. Scand J Dent Res. 1990 Aug;98 (4):351-5.

158. Munksgaard EC. Toxicology versus allergy in restorative dentistry. Adv Dent Res. 1992;6:17-21.

159. Scott A, Egner W, Gawkrodger DJ, Hatton PV, Sherriff M, van Noort R, et al. The national survey of adverse reactions to dental materials in the UK: a preliminary study by the UK Adverse Reactions Reporting Project. Br Dent J. 2004 Apr 24;196 (8):471-7; discussion 65.

160. Axéll T, Björkner B, Fregert S, Niklasson B. Standard patch test series for screening of contact allergy to dental materials. Contact Dermatitis. 1983 Jan;9 (1):82-4.

161. Lefebvre CA, Schuster GS, Rueggeberg FA, Tamareselvy K, Knoernschild KL. Responses of oral epithelial cells to dental resin components. J Biomater Sci Polym Ed. 1996;7 (11):965-76.

ERRATA

Paper I: After Paper I was published we discovered that Figs. 1 and 2 show peaks numbered incorrectly. The substance 25 should appear as one peak at retention time 16.33 exclusively and peak 26 at retention time 17.65 exclusively.

Paper I

Identification of organic eluates from four polymer-based dental filling materials

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Abstract

Elution from polymer-based dental filling materials may have a potential impact on the biocompatibility of the materials. Since information from the manufacturers about ingredients in the materials often is incomplete, analyses of eluates from the materials are necessary for a better knowledge about possible harmful compounds. The aim of this study was to identify organic eluates from polymerized samples of two composites, one compomer and one resin-reinforced glass ionomer cement. Samples were immersed in ethanol or Ringer's solution. Organic leachables were analyzed by gas chromatography–mass spectrometry. Identification was confirmed with reference substances, if available. Among components detected were monomers, co-monomers, initiators, stabilizers, decomposition products and contaminants. Thirty-two substances were identified and 17 were confirmed with reference substances. From elution in Ringer's we identified 13 eluates from Tetric Ceram, 10 from Z250, 21 from Dyract and six from Fuji II LC; HEMA, HC and CQ were found in all samples. From elution in ethanol 12 eluates from Tetric Ceram, 18 eluates from Z250, 19 from Dyract and 10 from Fuji II LCwere identified. The diversity of eluates from the four materials under study is demonstrated. Owing to variation between the materials, the biocompatibility including the allergenic potential may be different.

Key words: resin-based materials; leaching; residual monomers; analyses; additives

Paper II

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Quantification of organic eluates from polymerized resin-based dental restorative materials by use of GC/MS

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Abstract

Residual monomers, additives and degradation products from resin-based dental restorative materials eluted into the oral cavity may influence the biocompatibility of these materials. Emphasis has been placed on studies addressing cytotoxic, genotoxic and estrogenic potential of these substances. A prerequisite for analyzing the potential of exposure to eluted compounds from dental materials is reliable quantification methods, both real time and accelerated measurements. The purpose of the present study was to quantify nine eluates; 2-hydroxyethyl methacrylate (HEMA), hydroquinone monomethyl ether (MEHQ), camphorquinone (CQ), butylated hydroxytoluene (BHT), ethyl 4-(dimethylamino)benzoate (DMABEE), triethylene glycoldimethacrylate (TEGDMA), trimethylolpropane trimethacrylate (TMPTMA), oxybenzone (HMBP) and drometrizole (TIN P) leaching from specimens of four commonly used resin-based dental materials in ethanol and an aqueous solution. All analyses were performed by use of GC/MS, each component was quantified separately and the results presented in $\mu g\,mm^{-2}$. This study has shown that elution from various materials differs significantly, not only in the types of eluates, but also regarding amounts of total and of single components. A high amount of HMBP, a UV stabilizer with potential estrogenic activity, was detected from one material in both solutions.

Keywords: Quantification; Monomers; Gas chromatography/mass spectrometry; Resin-based dental composites; Eluates

1. Introduction

Dental resin-based restorative materials are complex polymers containing a variety of monomers, initiators, activators, stabilizers, plasticizers and other additives. The main organic ingredients are large monomers which during polymerization crosslink with smaller monomers to create a rigid polymer network. As the crosslinking propagates, diffusion inside the network is restricted, and complete cure is therefore not possible to achieve. The residual monomers and additives that are not chemically bond to the network are free to diffuse out from the cured materials. Several studies have shown that many of these compounds are leaching from the filling materials even after adequate polymerization [1–7]. It is known that some of the ingredients in the resin-based materials have cytotoxic [8–14], genotoxic [12,13] or allergenic effects [15–18] and/or exhibit estrogenic activity *in vitro* [19,20]. By use of chromatographic and mass spectrometric techniques, monomers and additives have been identified in aqueous and alcohol extracts of polymerized dental fillings [1–6,21]. In most studies, a limited number of compounds (mainly monomers) are quantified, few materials are investigated, and the results are sometimes contradictory. This might be explained by the fact that the quantitative results are obtained with different methods and presented in different ways. It is therefore difficult to compare various materials and the amount of single ingredients that can be extracted. The most extensive study was performed by Spahl et al. [2], who studied a large number of compounds eluted into water and methanol from four different resin-based materials. The various eluted compounds were not quantified separately, but their response in gas chromatograph/mass spectrometer (GC/MS) or liquid chromatograph/particle beam interface/mass spectrometer (LC/PB/MS) was compared to the response of caffeine.

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The adverse potential of leachables and degradation products, and the stability of the materials *in vivo*, is essential to consider the safety of a dental material. To evaluate the exposure of elutes to the human body, we need information about elution pattern and toxicokinetic factors, as well as reliable methods to measure the release of eluates. Both real time and accelerated measurements provides useful information about exposure in dose and time.

Recently we identified 32 eluates from four different resin-based dental filling materials [5]. The purpose of the present study was to quantify nine different leachables representing the various groups of ingredients; monomers, initiators, accelerators, inhibitors and stabilizers, from four different resin-based dental restorative materials (two composites, one compomer and one resin modified glass ionomer cement). The analytes were extracted into in ethanol and an aqueous solution and the quantitative results are presented as $\mu g/mm^2$ of specimen surface of the dental material.

2. Materials and methods

2.1. Standards and solvents

All standards listed in Table 1 were of analytical grade and obtained from Sigma–Aldrich, Oslo, Norway. Diethyl phthalate was purchased from Merck-Schuchardt, Hohenbrunn bei Munchen, Germany, and used as internal standard (I.S.). Ethanol was obtained from Arcus, Bergen, Norway. Ethyl acetate, NaOH and HCl were obtained from Merck, Darmstadt, Germany. All solvents and diethyl phthalate were checked to ensure they contained no compounds interfering with the analysis.

2.2. Preparation of specimens

Four different resin-based dental restorative materials were investigated (Table 2). The applied leaching model has previously been described in detail by Michelsen et al. [5].

Cylindrical stainless steel moulds were filled with uncured material to produce specimens with a diameter of 6 mm and a thickness of 2 mm. Care was taken to avoid air bubbles. Fourteen specimens of each material were prepared. The uncured materials were covered with a polyester film (Odus universal-strips, Odus Dental AG) and a glass plate to exclude the oxygen-inhibiting layer, and were polymerized by visible light with an Optilux 400 curing lamp (Demetron Research Corp., Danbury, CT, USA). The 14 specimens of each material were cured for 40 s. The light intensity was measured to be above 350 mW/cm^2 by a Curing Radiometer Model 100 (Demetron Research Corp.). Polymerization time of 40 s was in agreement with specification from the manufacturer for TC, DY and FU. For FZ, the manufacturer recommended a polymerization time of 20 s. However, after pilot studies we decided to apply the same curing time for all the materials.

Specimens were immersed in ethanol or Ringer's solution (9.0 g NaCl, 0.42 g KCl, 0.25 g $CaCl_2 \cdot 2H_2O$, in distilled water, total volume 1 l, pH adjusted to 7 with NaOH or HCl). Two series of seven glass tubes were prepared, one set with each glass tube containing 3 ml of Ringer's solution, and one parallel set with each glass tube containing 5 ml ethanol. The cured specimens were detached from the stainless steel moulds, and seven parallel specimens from each resin-based material were immediately immersed in Ringer's solution in the separate glass tubes. An identical series of seven parallel specimens was immersed likewise separately in the glass tubes containing ethanol. The glass tubes were secured with a ground glass stopper to prevent evaporation, and kept at 37 °C with constant agitation (200 rpm).

2.3. Specimens in ethanol

After 24 h, 1 ml of each ethanol solution was transferred to separate 10.5 ml glass vials (Karl Hecht, Germany), each vial

Table 1
The following authentic reference substances were used

Eluate	Cas nr	Mol. formula	Trivial name	Monoisotopic MW
HEMA	868-77-9	$C_6H_{10}O_3$	2-Hydroxyethyl methacrylate	130.1
MEHQ	150-76-5	$C_7H_8O_2$	4-Methoxyphenol (mequinol)	124.1
CQ	10373-78-1	$C_{10}H_{14}O_2$	(±)-Camphorquinone	166.1
BHT	128-37-0	$C_{15}H_{24}O$	Butylated hydroxytoluene	220.2
DMABEE	10287-53-3	$C_{11}H_{15}NO_2$	Ethyl 4-(dimethylamino)benzoate	193.1
TEGDMA	109-16-0	$C_{14}H_{22}O_6$	Triethyleneglycol dimethacrylate	286.1
TMPTMA	3290-92-4	$C_{18}H_{26}O_6$	Trimethylolpropane trimethacrylate	338.2
HMBP	131-57-7	$C_{14}H_{12}O_3$	2-Hydroxy-4-methoxybenzophenone (oxybenzone)	228.1
TIN P	2440-22-4	$C_{13}H_{11}N_3O$	2-(2-Hydroxy-5-methylphenyl) benzotriazole (drometrizole)	225.1

Table 2
The four different resin-based dental restorative materials investigated

Abbreviation	Type of material	Product name	Specifications	Manufacturer
TC	Composite	Tetric Ceram	Color A3, lot B42131	Vivadent Ets. Schaan, Lichtenstein
FZ	Composite	3M™ Filtek™ Z250	Color A3, lot 19991122	3M Svenska AB, Sollentuna, Sweden
DY	Compomer	Dyract AP	Color A3, lot 9909000451	Dentsply DeTrey GmbH, Konstanz, Germany
FU	Resin modified glass ionomer cement	GC Fuji II LC	Color A3, lot 080291	GC Corporation, Tokyo, Japan

containing 1 ml of ethyl acetate with an internal standard of diethyl phthalate (2 μg/ml). The solutions were evaporated to approximately 200 μl at 60 °C, and transferred to sample vials (Cromacol, London, UK).

2.4. Specimens in Ringer's solution

The specimens in Ringer's solution were removed from the glass tubes after 7 days. One millilitre of freshly distilled ethyl acetate with an internal standard (diethyl phthalate 2 μg/ml) was added to each of the seven parallel solutions, agitated for 1 min and rested. The solution from each glass tube was extracted three times with 2 ml of freshly distilled ethyl acetate and the extracts pooled for each sample. The seven pooled extracts were transferred to seven 10.5 ml glass vials (Karl Hecht, Germany), evaporated at 60 °C to approximately 200 μl and transferred to sample vials.

2.5. Separation by gas chromatography

The analyses were performed by using combined GC/MS. The instrument was a Thermo Quest Trace GC connected to a Finnigan MD 800 quadropol mass spectrometer. The GC was further equipped with an autosampler (Finnigan AS-800, Thermo Quest). For chromatographic separation, we used a capillary column with following specifications: CP-SIL 8 CB wall-coated open tubular (WCOT) low bleed fused silica MS column with length 30 m, i.d. of 0.25 mm and a film thickness of 0.25 μm (Chrompack, Middelburg, The Netherlands). The carrier gas was helium with a flow rate of 1 ml/min, constant flow. Splitless injection was used, injector temperature was 250 °C and purge flow of helium gas was 70 ml/min. The temperature program for the oven: start point at 50 °C, with a rate of 50 °C/min up to 120 °C, hold time at 120 °C for 5 min, from 120 to 280 °C with rate of 10 °C/min, hold time at 280 °C for 1 min. The syringe was rinsed with ethyl acetate five times before and after every injection. A hole with diameter of 3 mm was made in the rubber septum of the sample vial, to prevent septum particles to contaminate the sample following needle perforation. Evaporation of the sample was prevented with aluminum foil as a seal between the septum and the vial. The oven program and analyses were performed using the software package Xcalibur (Xcalibur™, Finnigan Corp.).

2.6. Mass spectrometric detection

Identification and quantification of the analytes were performed by using the mass spectrometer in full scan mode, scanning from 50 to 350 *m*/z. The identification of the different compounds was then based on comparison of the obtained full scan spectra with spectra in the NIST library (National Institute of Science and Technology, Gaithersburg, MD, USA), retention time (Table 3) and spectra of the reference substances. The quantifications were performed by constructing mass fragmentograms of abundant ions characteristic for each different analyte (Table 3), and comparing the area under each peak with the area of the internal standard peak.

2.7. Calibration curves

Calibration curves and response factors were computed with reference substances analyzed with the previously described method in five different concentrations for each compound; from 0.7 to 30 μg/ml with diethyl phthalate, 2 μg/ml, as internal standard.

2.8. Blanks and recovery

Ethanol, ethyl acetate and water were distilled twice to eliminate contaminants. The solvents were then subjected to the same treatment and extraction procedure as the samples, and analyzed by GC/MS. No peaks were found, and the blanks were considered to be without compounds interfering with the analysis. To avoid contamination from other polymer-based materials and plastics, gloves were not used, and all procedures were performed with metal instruments and glassware. Glassware and instruments were rinsed in distilled ethyl acetate twice, wrapped in aluminum foil and kept at 100 °C for at least 12 h before use. The foil was washed with ethyl acetate before wrapping the equipment. Foil and polyester film were tested for leachables, and no contaminating peaks were found.

The relative recovery of each analyte compared to the I.S. was tested with a solution of ethyl acetate with reference substances in concentrations 1 and 10 μg/ml and I.S. of 2 μg/ml. The ratios of amounts were compared before and after evaporation. Furthermore, reference substances in concentrations 1 and 10 μg/ml were added to Ringer's solution and measured after extraction and evaporation. The ratios to I.S. were compared to the ratios from the initial solution in ethyl acetate.

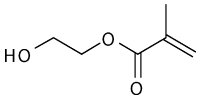
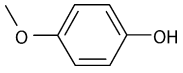
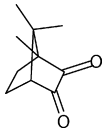
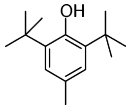
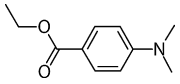
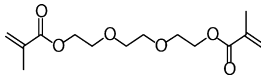
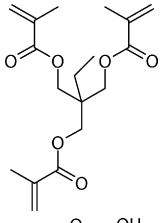
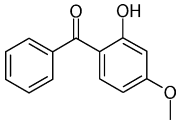
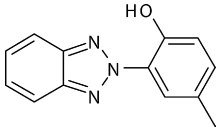
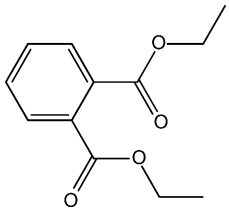
Lowest limit of detection, LOD, ≥3 S/N (signal to noise), and lowest limit of quantification, LOQ, ≥10 S/N, was found by analyzing reference compounds in concentrations from 0.001 to 10 μg/ml. Precision was tested with a reference cocktail in two concentrations, 1 and 10 μg/ml, and given as the standard deviation (S.D.) and relative standard deviation (R.S.D.) between repeated measurements for within-day and between-day measurements.

From the full scan spectra of each reference substance, one or two characteristic mass fragment was selected, preferably the base peak and/or the molecule ion (Table 3). The peak areas of these specific fragments in each sample of the reference substances were integrated and all integrations manually adjusted if necessary. Area ratios and response factors were incorporated in the calculation procedure, and the amounts of each eluate in each sample were computed.

3. Statistical methods

The results are presented as mean values with associated standard deviations (Fig. 2A and B). The Student's Independent-Samples *t*-test was used to test if

Table 3
Reference substances given with their function within the material, the molecular ion, characteristic ions, structure formula and retention time (The same parameters are given for I.S.)

Reference substance	Function	Molecular ion, *m/z*	Characteristic ions, *m/z*	Structure formula	RT (min)
HEMA	Monomer	130	69[a], 87		3.12
MEHQ	Inhibitor	124	109[a], 124		5.97
CQ	Initiator	166	95[a], 138, 166		8.21
BHT	Inhibitor	220	205[a], 220		11.71
DMABEE	Accelerator	193	148[a], 164, 193		14.60
TEGDMA	Monomer	286	69, 113[a]		16.33
TMPTMA	Monomer	338	69[a], 253		17.80
HMBP	UV stabilizer	228	151[a], 227		18.19
TIN P	UV stabilizer	225	225[a]		18.58
Internal standard:					
Diethyl phthalate	I.S.	222	149[a], 177		12.96

[a] Illustrates base peak.

observed differences in mean values of each compound were significant; in both solutions, and between each compound eluting from the various materials. The significance levels were expressed as two-tailed values and significance level was set at 0.05. The calculations were performed by using SPSS software (SPSS Inc., Chicago, USA).

4. Results and discussion

4.1. Materials

Composites, compomers and resin modified glass ionomer cements all contain an organic polymer matrix with inorganic filling particles embedded. Yet, ingredients differ greatly

Table 4
Eluates and given ingredients from the four materials

Eluates/ingredients	TC		FZ		DY		FU	
	Detected[a]	MSDS	Detected[a]	MSDS	Detected[a]	MSDS	Detected[a]	MSDS
HEMA	*x*		*x*		*x*		*x*	*x*
MEHQ	*x*		*x*		*x*		*x*	
CQ	*x*		*x*		*x*		*x*	*x*
BHT			*x*		*x*		*x*	*x*
DMABEE	*x*		*x*		*x*			
TEGDMA	*x*	*x*	*x*	*x*				
TMPTMA					*x*	*x*		
HMBP					*x*			
TIN P	*x*							

In the column marked MSDS, *x* represents ingredients given in the MSDS from the manufacturer.
[a] Represents eluates detected in amounts higher than limit of quantification.

concerning types and amounts [5]. The Material Safety Data Sheets (MSDS) are known to be incomplete and sometimes misguiding [22,23]. In the MSDS, the manufacturers are obliged to give information about the main ingredients (≥1%). Most additives and some monomers are present in concentrations below 1% and therefore information about these compounds is not given (Table 4). Furthermore, some compounds found in the materials are not purposely added by the producers, but are remnants from the synthesis of the raw materials, like stabilizers and catalysts, i.e. triphenyl antimony [2,3,5]. The exposure to components in minor amounts (less than 1%), however, cannot be excluded to be responsible for allergic or other adverse effects.

The weight percent of resin components in resin-based dental restorative materials is higher in composites than in compomers. Furthermore, the presence of resin components is lower in the resin modified glass ionomer materials. Monomers, the main organic ingredients, range from small molecules to high molecular weight substances. During polymerization the monomers crosslink to create a polymeric matrix. The polymerization is however, not complete, leaving up to 10% of residual monomers capable of leaching out [24]. Residual low molecular weight monomers, like HEMA and TEGDMA, are relatively mobile, and may diffuse through the matrix into an immersion medium. High molecular weight monomers like Bis-GMA (2,2-bis[4-(2′-hydroxy-3′-methacryloxypropoxy)phenyl]propane) are rigid and hydrophobic, and not likely to diffuse out from the materials [24]. The various additives, besides being of low molecular weight, are often not included in the polymerized network and are therefore easily eluted.

4.2. Immersion media

The type of immersion media has a substantial effect on the rate of elution of the elutable molecules. To simulate an oral environment, we have used Ringer's solution to extract the eluates from the cured specimens. Ringer's solution was used because it has previously been employed as a physiological saline solution [25]. Aqueous solvents are not able to extract the total amount of eluates from the cured specimens. Therefore, we have also used ethanol as an extracting solvent to be able to estimate the total amount of compounds that might leak from the actual filling materials. As expected, higher amounts of eluates were found in ethanol solutions compared to in Ringer's solutions, Fig. 2A and B. This is especially pronounced from the resin modified glass ionomer cement specimens. The choice of elution time was based on pilot studies, which demonstrated that elution in ethanol was close to completed within the first 24 h, whereas in Ringer's solution the elution was considered completed after 7 days. Ferracane and Condon found 75–100% of the leachable components to be eluted from composite specimens into ethanol within the first few hours, and component release was considered essentially complete after 24 h both in 75% ethanol and in water [26]. In some studies, the specimens are allowed to desiccate for 24 h to 7 days prior to immersion in media [27,28]. However, in our experiment the specimens were polymerized and immediately immersed in media, since this is clinically more relevant. A strong correlation between the surface area of the specimens and the amounts of eluted TEGDMA in short time elution was demonstrated by Pelka et al. [29]. This is the reason why we express the elution in μg/mm^2. In the study the mass/volume ratio between the specimens and the test solutions were at least 1:10, and the specimens were fully immersed in the test solutions according to ISO 10993-13 [30].

4.3. Evaporation, extraction and recovery

The extraction into ethyl acetate of eluatable compounds from Ringer's solution was performed three times. By use of GC/MS, we confirmed in pilot studies that eluates were present in the first and in the second extraction. The analysis of the third, fourth and fifth extract, however, displayed no detectable compounds; indicating that three extractions should be sufficient. Recovery test after extraction and evaporation, and evaporation exclusively, showed 72–103% and 86–107% recovery, respectively. This was performed for all analytes in two different concentrations, 1 and 10 μg/ml. In the analysis of specimens, it is possible that a portion of the detected HEMA might be a result of the decomposition of larger monomers like UEDMA (Urethane modified Bis-GMA). To confirm this, we analyzed standard samples of the most

common monomers in dental composites; UEDMA, Bis-EMA (2,2-bis[4-(2′-methacrylyloxyethoxy)phenyl]propane) and Bis-GMA and found minor amounts of HEMA decomposed from UEDMA, in accordance with findings of Spahl et al. [2,31].

4.4. GC/MS

The monomers in resin-based dental restorative materials range from low to high molecular weight (MW) substances. In the analysis of low MW monomers and additives GC/MS based methods are to be preferred [32]. Analysis of the high molecular weight monomers Bis-GMA, Bis-EMA and UEDMA are better performed by use of high performance liquid chromatography, HPLC [2,31]. All analytes investigated here can be classified as low MW compounds, therefore, all analysis were performed by the use of GC/MS.

Fig. 1 displays chromatograms of elution from the specimens immersed in Ringer's solution. All peaks were sharp and symmetrical, except from MEHQ, which gave broader peaks. Ethyl acetate was injected between the samples series from each material to check if there were any carry-over effects during analysis. No peaks were observed above LOD.

4.5. Limit of quantification and limit of detection

In our analysis, the limit of detection, LOD, varies between the different substances, and was between 0.01 and 1 μg/ml. Low weight molecules needed higher concentrations to be detected. Limit of quantification, LOQ, was between 0.1 and 1 μg/ml. Lower amounts can be detected by using the GC/MS in selected ion monitoring (SIM) mode. However, at this stage we wanted to ensure a reliable identification of the eluted compounds, therefore, the mass spectrometer was used in full scan mode.

4.6. Precision

Within-day precision between injections of reference substances was measured as the standard deviation (S.D.) from 0.150 to 0.002, and the relative standard deviation (R.S.D.) from 0.451 to 0.018%. Between-day variation calculated during 2 days, was between 0.159 and 0.003 (S.D.) and R.S.D. was between 0.512 and 0.019% for all compounds, with a slightly higher S.D. and R.S.D. for the higher concentrations investigated (Table 5).

4.7. Calibration curves

The linearity was good for all substances in our test concentrations. Furthermore, the concentrations of eluted substances were well inside the linearity range, except for TEGDMA from TC and TMPTMA from DY in ethanol which both eluted slightly higher concentrations. The concentrations could preferable have been calibrated up to 50 μg/ml. R^2 was 0.99 for HEMA, MEHQ, DMABEE, TMPTMA and TIN P, 0.98 for BHT and TEGDMA, 0.94 for HMBP and 0.76 for CQ. The low R^2 for CQ is probably due to low sensitivity for this compound.

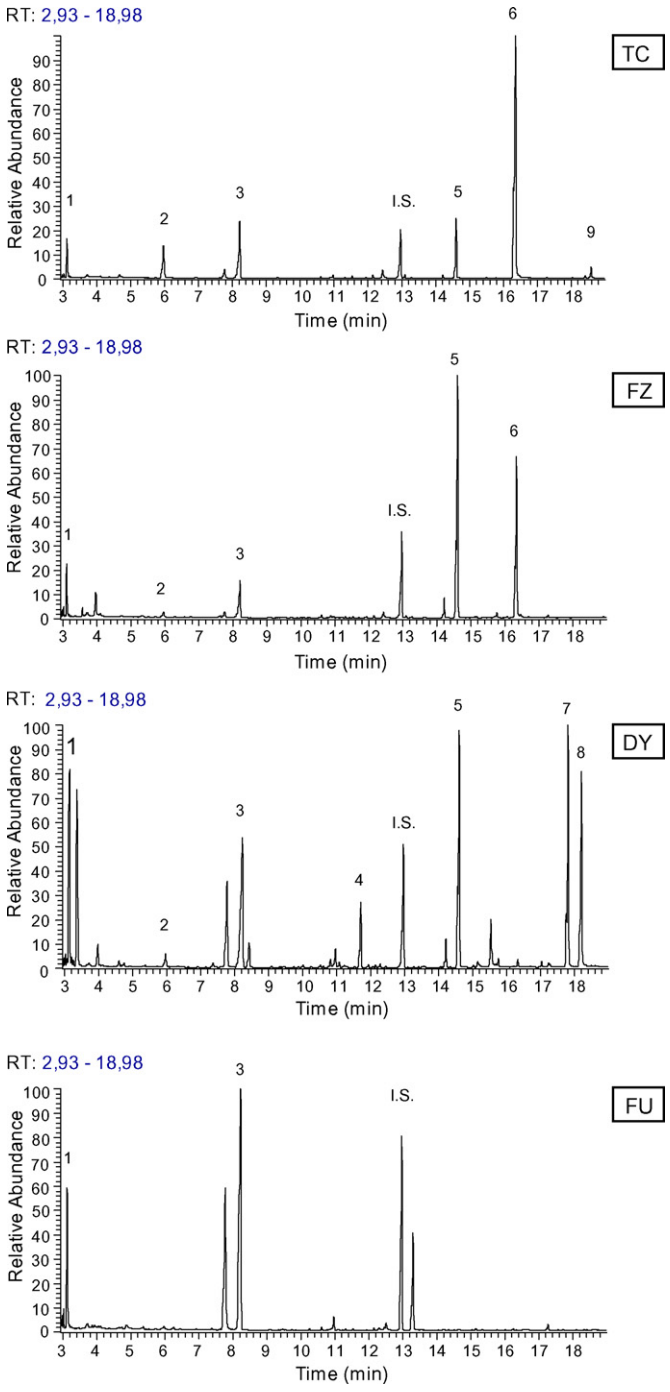


Fig. 1. Chromatograms of elution in Ringer's solution from TC, FZ, DY and FU. Sections from RT 3 to 19 are displayed. 1 = HEMA, 2 = MEHQ, 3 = CQ, 4 = BHT, 5 = DMABEE, 6 = TEGDMA, 7 = TMPTMA, 8 = HMBP, 9 = TIN P.

4.8. Internal standard

In previous studies on resin-based materials, monomers have been quantified by using external standard curves with reference substances [28,33–37]. Very few reports have applied internal standards (I.S.) in the quantification procedure [2,21,27,32]. In two studies caffeine was used as I.S. [2,32] and the amounts of elutable compounds was given as a percentage of the response of caffeine. Caffeine may be chosen because of a suitable reten-

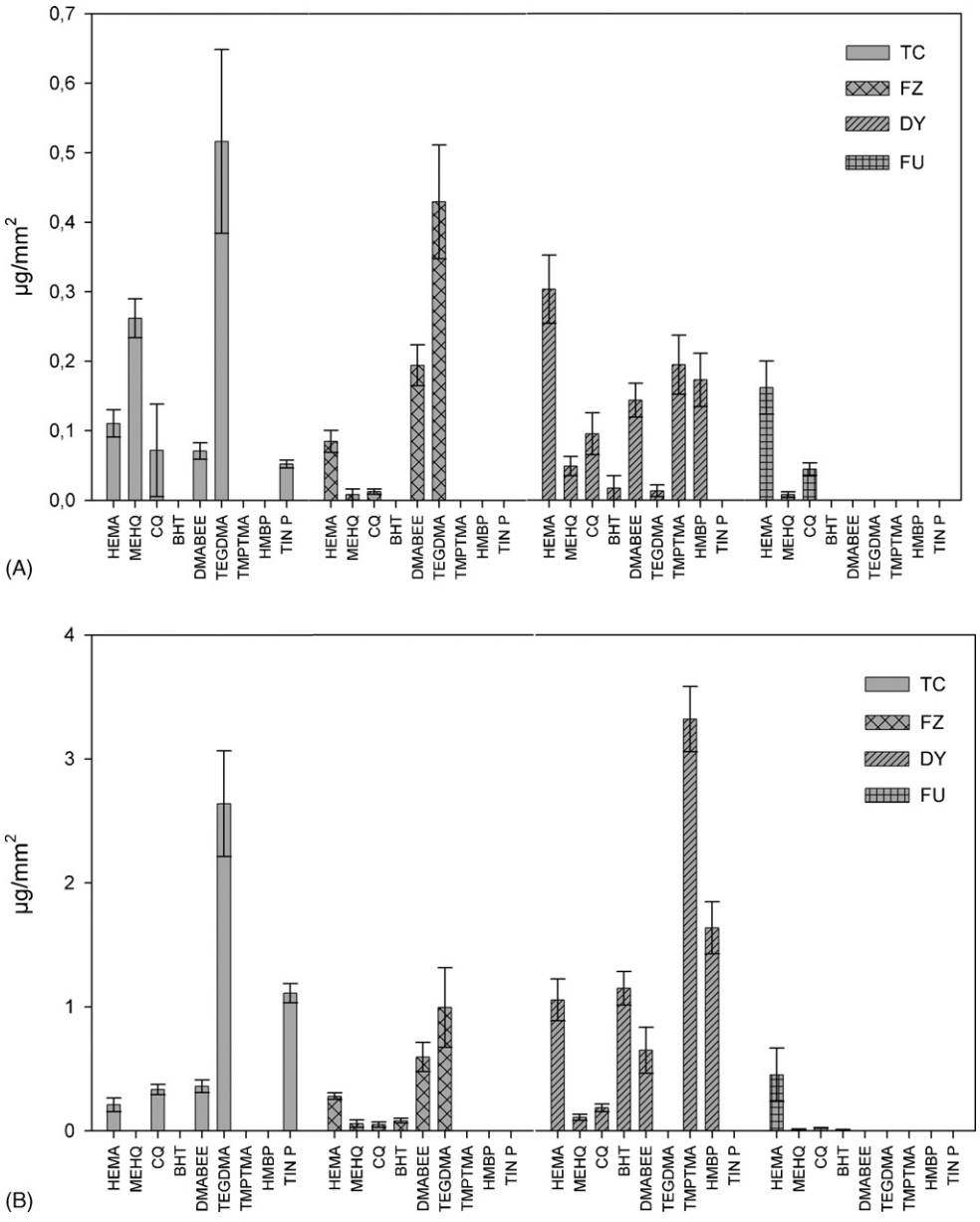


Fig. 2. Eluates from Ringer's solution (A) and from ethanol (B), quantities given in µg/mm^2 of sample surface. Mean values (bars) and S.D. (vertical lines) are given.

tion time. However, for *in vivo* analysis of saliva the potential of caffeine as a contaminating substance is high. In our study, we planned for further *in vivo* analysis, and accordingly, diethyl phthalate was used as an I.S. Diethyl phthalate has a high stability, easily detectable mass fragments and a retention time which is not interfering with the elutable compounds. Stable isotopes labelled standards would be most preferable for the quantification procedure [38], because they behave identically during sample preparation, extraction and evaporation. Deuterated or ^{13}C labelled analogues were, however, not available for the nine substances in this study.

4.9. Quantities of eluates from specimens

The quantities of eluted compounds, showed a wide variation depending on the elution media as shown in Fig. 2A and B. In ethanol, the eluted amounts was statistically significantly higher compared to eluted amounts in Ringer's solution for all substances ($p < 0.05$) except for MEHQ from TC. The highest observed difference was measured from TC for the compound TIN P for which the eluted amount was 20 times higher in ethanol compared to in Ringer's solution. Monomers represented the dominating group of elutable compounds, in ethanol as in Ringer's solution. The highest amount of a single substance (TMPTMA) eluted from one specimen was 3.28 µg/mm^2 specimen surface, eluted in ethanol from DY.

However, the most interesting findings are the differences in amounts of eluted compounds depending on the composition of the materials. The variation of eluted amounts in Ringer's solution, which is clinically most relevant, is shown in Fig. 2A. The mean value of TEGDMA was higher in TC than in FZ, and lower from DY. However, the difference in detected amounts of

Table 5
Precision of the assay for all nine analytes in two concentrations for within-day and between-day measurements

Precision of the assay[a]					
Analyte		Within-day		Between-day	
		S.D.	R.S.D. (%)	S.D.	R.S.D. (%)
HEMA	10 μg/ml	0.050	0.451	0.055	0.512
	1 μg/ml	0.016	0.070	0.011	0.049
MEHQ	10 μg/ml	0.024	0.236	0.074	0.297
	1 μg/ml	0.018	0.038	0.012	0.042
CQ	10 μg/ml	0.003	0.294	0.074	0.369
	1 μg/ml	0.010	0.043	0.012	0.047
BHT	10 μg/ml	0.017	0.156	0.021	0.153
	1 μg/ml	0.002	0.318	0.003	0.335
TEGDMA	10 μg/ml	0.062	0.079	0.074	0.091
	1 μg/ml	0.029	0.045	0.033	0.053
TMPTMA	10 μg/ml	0.150	0.018	0.159	0.019
	1 μg/ml	0.054	0.029	0.051	0.121
DMABEE	10 μg/ml	0.009	0.169	0.011	0.196
	1 μg/ml	0.005	0.138	0.005	0.158
HMBP	10 μg/ml	0.074	0.202	0.084	0.220
	1 μg/ml	0.030	0.162	0.030	0.164
TIN P	10 μg/ml	0.093	0.196	0.107	0.216
	1 μg/ml	0.045	0.178	0.046	0.180

[a] $n = 15$ for within-day precision and $n = 10$ for between-day precision for all concentrations.

TEGDMA in TC and FZ was not statistically significant. From FU no TEGDMA was found, on the other hand HEMA was the dominating monomer. The mean values of eluted HEMA were as follows: DY > FU > TC > FZ. The observed differences were statistically significant.

From the initiator system, the eluted DMABEE in Ringer's solution showed mean values from FZ > DY > TC. The observed differences were all statistically significant. Compared to TC, FZ eluted only about 25% of the amount of CQ in Ringer's solution. This might partly be explained by the presence in FZ of an additional initiator, di-phenyliodonium chloride (DPICl) [5]. DPICl in addition to TEGDMA, has been assumed to be the reason for the cytotoxic effect of the resin modified glass ionomer cement, Vitrebond [32]. Thus, the potential effect of each eluate has to be considered, not only the highest total amounts of eluates. Furthermore, the eluate mixture might also be of importance; in a resent study a higher experimental cell death inducing potential was indicated from mixtures of monomers than from the single monomers [14].

HMBP, an UV stabilizer, found to leach from the compomer (DY) in this study, was recently found to have estrogenic activity *in vitro* [39] in concentrations above 1 μmol/l (0.23 μg/ml). The mean amount found in our study was 0.17 $\mu g/mm^2$ eluted from DY specimens in Ringer's solution. From elution in ethanol we measured about ten times this amount (1.64 $\mu g/mm^2$) as an indication of possible maximum elution potential. The *in vivo* implication of the detected amounts of HMBP is difficult to assess. However, the results may indicate that the potential of estrogenic activity cannot be excluded. High quantities of this compound and potential long term biological effects should be carefully considered.

5. Conclusions

The applied GC/MS method seems well suited for analysis of small monomers and additives eluting from composites, compomers and resin modified glass ionomer cements. The analyzed eluates included several groups of ingredients; monomers, initiators, accelerators, inhibitors and stabilizers. The results allowed for a possibility to compare eluted amounts of organic compounds between various resin-based dental restorative materials. Our study has shown that the elution pattern from resin-based dental restorative materials differs, not only in the types of eluates, but also regarding the total and single components' amounts. For that reason the materials have different potential for causing adverse effects, thus in assessment of biocompatibility both quantitative and qualitative evaluations of the eluates has to be considered. Since the health hazard of several of the substances leaching is questioned, better knowledge of the exposure to the human body is important.

When an allergic reaction from an ingredient of these materials is revealed, better knowledge about the composition of the products may give an opportunity to select an appropriate filling material in clinical dentistry.

The results from this study represent *in vitro* elution in ethanol and Ringer's solution. Eluted amounts from resin-based dental restorative materials into human saliva might be quantified somewhere in between. In the oral environment, the process of leaching is affected by many factors, such as saliva's composition, pH and the amount of saliva secretion. Further studies will be addressed to *in vivo* situations by collecting saliva samples that has been in contact with resin-based dental restorative materials.

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References

[1] W. Geurtsen, Eur. J. Oral. Sci. 106 (1998) 687.
[2] W. Spahl, H. Budzikiewicz, W. Geurtsen, J. Dent. 26 (1998) 137.
[3] S.Y. Lee, H.M. Huang, C.Y. Lin, Y.H. Shih, J. Oral. Rehabil. 25 (1998) 575.
[4] H. Lygre, P.J. Høl, E. Solheim, G. Moe, Eur. J. Oral. Sci. 107 (1999) 378.
[5] V.B. Michelsen, H. Lygre, R. Skålevik, A.B. Tveit, E. Solheim, Eur. J. Oral. Sci. 111 (2003) 263.
[6] R. Rogalewicz, K. Batko, A. Voelkel, J. Environ. Monit. 8 (2006) 750.
[7] R. Rogalewicz, A. Voelkel, I. Kownacki, J. Environ. Monit. 8 (2006) 377.
[8] W. Geurtsen, F. Lehmann, W. Spahl, G. Leyhausen, J. Biomed. Mater. Res. 41 (1998) 474.
[9] S. Bouillaguet, J.C. Wataha, C.T. Hanks, B. Ciucchi, J. Holz, J. Endod. 22 (1996) 244.

[10] C.T. Hanks, S.E. Strawn, J.C. Wataha, R.G. Craig, J. Dent. Res. 70 (1991) 1450.
[11] T. Atsumi, J. Murata, I. Kamiyanagi, S. Fujisawa, T. Ueha, Arch. Oral. Biol. 43 (1998) 73.
[12] J. Heil, G. Reifferscheid, P. Waldmann, G. Leyhausen, W. Geurtsen, Mutat. Res. 368 (1996) 181.
[13] H. Schweikl, I. Altmannberger, N. Hanser, K.A. Hiller, C. Bolay, G. Brockhoff, G. Spagnuolo, K. Galler, G. Schmalz, Biomaterials 26 (2005) 4111.
[14] R. Becher, H.M. Kopperud, R.H. Al, J.T. Samuelsen, E. Morisbak, H.J. Dahlman, E.M. Lilleaas, J.E. Dahl, Dent. Mater. 22 (2006) 630.
[15] A. Hensten-Pettersen, Eur. J. Oral. Sci. 106 (1998) 707.
[16] U. Örtengren, H. Andreasson, S. Karlsson, B. Meding, L. Barregård, Eur. J. Oral. Sci. 107 (1999) 496.
[17] P. Collins, J. Ferguson, Br. J. Dermatol. 131 (1994) 124.
[18] B. Niklasson, B. Björkner, Contact Dermat. 21 (1989) 330.
[19] N. Olea, R. Pulgar, P. Perez, F. Olea-Serrano, A. Rivas, A. Novillo-Fertrell, V. Pedraza, A.M. Soto, C. Sonnenschein, Environ. Health Perspect. 104 (1996) 298.
[20] J.B. Lewis, F.A. Rueggeberg, C.A. Lapp, J.W. Ergle, G.S. Schuster, Clin. Oral Investig. 3 (1999) 107.
[21] S.A. Mazzaoui, M.F. Burrow, M.J. Tyas, F.R. Rooney, R.J. Capon, J. Biomed. Mater. Res. 63 (2002) 299.
[22] L. Kanerva, M.L. Henriks-Eckerman, R. Jolanki, T. Estlander, Clin. Dermatol. 15 (1997) 533.
[23] M.L. Henriks-Eckerman, K. Suuronen, R. Jolanki, K. Alanko, Contact Dermat. 50 (2004) 233.
[24] J.L. Ferracane, J. Oral Rehabil. 21 (1994) 441.
[25] Y. Takahashi, J. Hasegawa, Y. Kameyama, Dent. Mater. 5 (1989) 256.
[26] J.L. Ferracane, J.R. Condon, Dent. Mater. 6 (1990) 282.
[27] H. Müller, S. Olsson, K.-J. Söderholm, Eur. J. Oral Sci. 105 (1997) 362.
[28] E.C. Munksgaard, A. Peutzfeldt, E. Asmussen, Eur. J. Oral Sci. 108 (2000) 341.
[29] M. Pelka, W. Distler, A. Petschelt, Clin. Oral Investig. 3 (1999) 194.
[30] International standard ISO 10993-13 (1998).
[31] W. Spahl, H. Budzikiewicz, Fresenius, J. Anal. Chem. 350 (1994) 684.
[32] W. Geurtsen, W. Spahl, G. Leyhausen, J. Dent. Res. 77 (1998) 2012.
[33] U. Örtengren, S. Langer, A. Göransson, T. Lundgren, Eur. J. Oral Sci. 112 (2004) 530.
[34] K. Tanaka, M. Taira, H. Shintani, K. Wakasa, M. Yamaki, J. Oral Rehabil. 18 (1991) 353.
[35] A. Hamid, A. Okamoto, M. Iwaku, W.R. Hume, J. Oral Rehabil. 25 (1998) 94.
[36] K. Kawai, T. Takaoka, Am. J. Dent. 15 (2002) 149.
[37] J.G. Kim, C.M. Chung, J. Biomed. Mater. Res. B Appl. Biomater. 72 (2005) 328.
[38] P.I. Holm, P.M. Ueland, G. Kvalheim, E.A. Lien, Clin. Chem. 49 (2003) 286.
[39] H. Wada, H. Tarumi, S. Imazato, M. Narimatsu, S. Ebisu, J. Dent. Res. 83 (2004) 222.

Paper III

DENTAL MATERIALS XXX (2007) XXX–XXX

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Quantitative analysis of TEGDMA and HEMA eluted into saliva from two dental composites by use of GC/MS and tailor-made internal standards

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ABSTRACT

Objectives. The use of resin-based dental restorative materials is rapidly increasing, concurrently the biocompatibility of the materials is under investigation. Attention has been placed on studies addressing the cytotoxic, genotoxic and estrogenic potential of these materials. Therefore, the degree of exposure to eluted compounds from the dental materials is of high interest. The aim of this study was to assess the amounts of 2-hydroxyethyl methacrylate (HEMA) and triethyleneglycol dimethacrylate (TEGDMA), released from two composites, eluting into human saliva. To improve the method of quantification, three tailor-made internal standards were synthesized.

Methods. Specimens made from two composites (Tetric EvoCeram and Filtek Z250) were polymerized and immersed in human saliva for 24 h. Eluted TEGDMA and HEMA were identified and quantified. The quantitative analyses were performed by use of combined gas chromatography–mass spectrometry (GC/MS) with tailor-made internal standards synthesized by dissolving HEMA or TEGDMA in methanol and reducing the double bond of the methacrylate group by hydrogenation with $^1H_2$ and $^2H_2$ (D_2) gas.

Results. HEMA was released from both materials, whereas TEGDMA eluted from Filtek Z250 only. Full scan GC–MS analysis of each tailor-made internal standard demonstrated one peak only, which was well separated from the corresponding analyte's peak and with no traces of HEMA or TEGDMA.

Significance. The quantification method seems well suited for *in vivo* analysis, and the three standards synthesized represent an improved tool for quantification of the eluted monomers. The synthesis may be applied to other methacrylate monomers to produce tailor-made standards for quantification.

1. Introduction

Triethyleneglycol dimethacrylate (TEGDMA) and 2-hydroxyethyl methacrylate (HEMA) are common monomers in resin-based dental restorative materials. Several reports have shown that TEGDMA and HEMA are released from the polymerized materials [1–4]. Concern about the biocompatibility and potential adverse effects has initiated studies that found HEMA and

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Table 1 – Cas numbers, molecular formula, trivial name, structure and molecular weight of reference substances and internal standards

Substance	Cas no.	Mol. formula	Trivial name	MW
HEMA	868-77-9	$C_6H_{10}O_3$	2-Hydroxyethyl methacrylate	130
TEGDMA	109-16-0	$C_{14}H_{22}O_6$	Triethyleneglycol dimethacrylate	286
DEP	84-66-2	$C_{12}H_{14}O_4$	Diethyl phthalate	222
IS-HD	N.A.	$C_6H_{10}D_2O_3$		134
IS-TD	N.A.	$C_{14}H_{22}D_4O_6$		294
IS-TH	N.A.	$C_{14}H_{26}O_6$		290

TEGDMA to be cytotoxic [5,6] and allergenic [7], and furthermore TEGDMA to be genotoxic also [8]. These monomers are described to induce apoptosis *in vitro* [9–11]. TEGDMA has been found to be toxic to dental pulp and to human gingival fibroblasts [12]. Furthermore, TEGDMA has been suggested to be a contributor to dental resin-induced adverse effects, associated with a depletion of intracellular glutathione [13,14]. It has been hypothesized that resin monomers may induce production of reactive oxygen species (ROS) causing cellular stress [12,15]. In addition, TEGDMA has been found to have adverse effects on the fertility and the reproductive system of male mice [16]. Apoptosis and necrosis induced by TEGDMA and HEMA has been demonstrated to be concentration dependent [9]. Thus, a reliable method for quantification of the monomers released into saliva may contribute to the knowledge about potential hazards posed by the resin-based dental restorative materials.

In previous studies we have characterized eluates from four resin-based dental restorative materials by identification and quantification [3,17]. The aim of the present study was to quantify HEMA and TEGDMA eluting from polymerized composite materials into saliva, and to improve earlier developed quantification methods. We used human saliva as an immersion medium to mimic an *in vivo* situation and to prepare for further *in vivo* studies. Eluted TEGDMA and HEMA were identified and quantified by use of combined gas chromatography–mass spectrometry (GC/MS) and tailor-made internal standards synthesized in our laboratory.

2. Materials and methods

2.1. Chemicals

HEMA and TEGDMA were of analytical grade and obtained from Sigma–Aldrich, Oslo, Norway, details are shown in Table 1. Diethyl phthalate was purchased from Merck-Schuchardt, Hohenbrunn bei Munchen, Germany. Methanol, ethyl acetate and the catalyst 10% Pd/charcoal was obtained from Merck, Darmstadt, Germany. The standards and diethyl phthalate were checked with full scan GC/MS to ensure they

Table 2 – The two different resin-based dental restorative materials investigated

Abbreviations	Type of material	Product name	Specifications	Manufacturer
TEC	Composite	Tetric EvoCeram	Color A2, lot H21573	Ivoclar Vivadent AG, FL-9494 Schaan/Liechtenstein
FZ	Composite	3M ESPE Filtek™ Z250	Color A2, lot 20051213	3M ESPE Dental Products, St. Paul, MN 55144-1000, USA

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contained no compounds interfering with the analysis. $^{1}H_1$ and $^{2}H_2$ gas was purchased from Yara, Norway. The filter substance Celite 545 was from Kebolab, Stockholm, Sweden.

2.2. Preparation of samples

Two different dental composites were investigated (Table 2). The applied leaching model has previously been described in detail by Michelsen et al. [3,17]. Cylindrical teflon moulds were filled with uncured material to produce samples with a diameter of 6 mm and a thickness of 2 mm. Care was taken to avoid air bubbles. Nine samples of each material were prepared. The uncured materials were covered with a polyester film (Odus universal-strips) and a glass plate to exclude the oxygen-inhibiting layer, and were polymerized by visible light with an Optilux 400 curing lamp (Demetron Research Corp., Danbury, CT, USA). The nine specimens of each material were cured according to the manufacturer; 20 s for both materials. The light intensity was measured to be above 500 mW cm^{-2} by a Curing Radiometer model 100 (Demetron Research Corp.). The mean weight of the samples was 0.1434 ± 0.0022 g (relative standard deviation (R.S.D.) was 3.76%). Samples were immediately immersed in saliva. The saliva was non-stimulated and collected from one individual with no dental restorations. Two hours before the saliva collection tooth brushing and interdental hygiene was performed, furthermore from this point on, no intake of food or liquids (except water) and no smoking were allowed until sampling was finished. No intake of alcohol was allowed the day before sampling. After collection the saliva was frozen at −28 °C. GC/MS analysis of the collected saliva revealed no compounds interfering with the analysis. To avoid contamination from other polymer-based materials and plastics during sampling and further throughout the study, gloves were not used, and all procedures were performed with metal instruments and glassware. Glassware and instruments were wrapped in aluminum foil and heated at 400 °C for 4 h in a muffle furnace (Carbolite CWF 1200). Ethyl acetate was distilled twice, and analyzed by GC/MS to ensure it contained no compounds interfering with the analysis. Furthermore, foil and polyester film were tested for contaminating leachables.

Two series of glass tubes were prepared, all with each glass tube containing 1 ml of saliva. The cured samples were detached from the teflon moulds, and the nine parallel samples from each resin-based dental filling material were immediately immersed in saliva in the separate glass tubes. The glass tubes were secured with a ground glass stopper to prevent evaporation, and kept at 37 °C with agitation. After 24 h, the specimens were removed and the saliva solutions were transferred to separate 10.5 ml glass vials (Karl Hecht, Germany), each vial containing one ml of freshly distilled ethyl acetate with 1.6 μg of the internal standard IS-TD (Table 1) and 1.25 μg of IS-HD (Table 1). Furthermore, these solutions were each added 1 ml of freshly distilled ethyl acetate with an internal standard of diethyl phthalate (2 μg ml^{-1}), agitated for 1 min and rested. From each vial the leachables were extracted three times with 2 ml of distilled ethyl acetate and the extracts pooled for each sample. The nine pooled extracts were transferred to 10.5 ml glass vials, evaporated at 53 °C to approximately 200 μl and transferred to sample vials (Chromacol, London, UK) for further analysis.

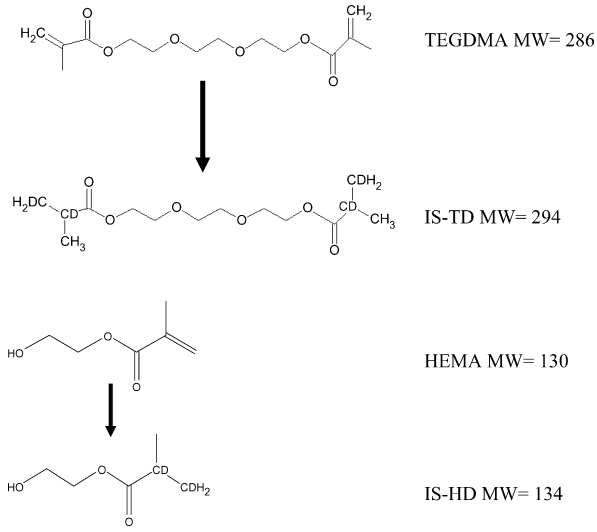

Fig. 1 – Synthesis of tailor-made internal standards.

The analogue internal standard IS-TD was synthesized as follows: TEGDMA (0.5 ml, 1.91 mmol) was dissolved in MeOH (15 ml) and reduced by hydrogenation with D_2 gas on 10% Pd/charcoal at atmospheric pressure for 2 h, according to Fig. 1. The reaction product was filtered through a short column filled with Celite 545 and the compound synthesized was analyzed by GC/MS in full scan mode. The same procedure was carried out with HEMA to synthesize IS-HD. For synthesis of IS-TH we used the same method but with hydrogenation with $^{1}H_2$ gas instead of $^{2}H_2$ gas.

2.3. Analytical procedure

The analyses were performed by using combined GC/MS. The instrument was a Thermo Quest Trace GC connected to a Finnigan MD 800 quadrupole mass spectrometer. The GC was further equipped with an auto sampler (Finnigan AS-800, Thermo Quest). For chromatographic separation we used a capillary column with following specifications: CP-SIL 8 CB wall-coated open tubular (WCOT) low bleed fused silica MS column with column length 30 m, i.d. of 0.25 mm and a film thickness of 0.25 μm (Chrompack, Middelburg, The Netherlands). The carrier gas was helium with a flow rate of 1 ml min^{-1}, constant flow. Split less injection was used; injector temperature of 250 °C, purge flow of helium gas was 70 ml min^{-1}. The temperature program for the oven: start point at 50 °C, with a rate of 50 °C min^{-1} up to 120 °C, hold time at 120 °C for 5 min, from 120 to 230 °C with rate of 10 °C min^{-1}, 230 to 280 °C with rate of 120 °C min^{-1} hold time at 280 °C for 1 min. The syringe was rinsed with ethyl acetate five times before and after every injection. A hole with diameter of 3 mm was made in the rubber septum of the sample vial, to prevent septum particles to contaminate the sample following needle perforation. Evaporation of the sample was prevented with aluminum foil as a seal between the septum and the vial. The oven program and analyses were performed using the software package Xcalibur (Xcalibur™, Finnigan Corp.).

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Table 3 – Qualifying ions and base peaks for SIM analyses of eluates and internal standards

Substance	Qualifying ions	Base peak
TEGDMA	113	69
IS-TH	71	115
IS-TD	73	117
HEMA	87	69
IS-HD	91	73
DEP, diethyl phthalate	149	177

Identification of the analytes was performed by using the mass spectrometer in full scan mode. The mass range for detection was 50–350 *m/z*. The analytes were identified by comparing the mass spectra with the National Institute of Science and Technology (NIST, Gaithersburg, MD, USA) mass spectra library. Finally, retention time (RT) and mass spectra of the eluates were compared with that of authentic reference substances (Table 1). For each reference substance and the internal standards, one or two characteristic mass fragments were selected (Table 3). After identification, analyses for quantification were performed in the SIM mode, recording fragments according to Table 3. The peaks of these specific fragments in each sample of the reference substances were integrated and the areas were compared with internal standard areas. All integrations were manually adjusted if necessary. Standard curves and response factors were computed. Linearity of area ratios versus quantities was confirmed for all substances.

Reference substances were analyzed with the previously described method in five different concentrations; from 0.2 to 10 $\mu g\,ml^{-1}$. Lowest limit of detection, LOD $\geq$ 3 S/N (signal to noise), and lowest limit of quantification, LOQ, $\geq$10 S/N, was found by analyzing reference compounds in concentrations from 0.002 to 2 $\mu g\,ml^{-1}$. Accuracy and precision was tested with a reference cocktail in two concentrations, 1 and 5 $\mu g\,ml^{-1}$, and given as the standard deviation (S.D.) and relative standard deviation between repeated measurements for within-day and between-day measurements. The relative recovery of HEMA and TEGDMA compared to diethyl phthalate was tested previously with a solution of ethyl acetate with reference substances in concentrations 1 and 10 $\mu g\,ml^{-1}$ and diethyl phthalate of 2 $\mu g\,ml^{-1}$ [17]. The ratios of amounts were compared before and after evaporation, and before and after extraction from water, and found satisfactory. Accuracy was tested with five different concentrations; from 1 to 10 $\mu g\,ml^{-1}$, and given as standard deviation and relative standard deviation.

2.4. *Statistical methods*

The results are presented as $\mu g\,cm^{-2}$ eluted from the surface of a composite sample, and expressed as mean values with associated standard deviations. The Student's independent-samples *t*-test was used to test if observed differences in mean values of each compound eluting from the two materials were significant. The significance levels were expressed as two-tailed values and significance level was set at 0.05. The calculations were performed by using SPSS software (SPSS Inc., Chicago, USA).

2.5. *Ethical committee*

The study is part of a study on leachables from resin-based restorative materials in human saliva. This study and an associated establishment of a research bio bank for saliva collected in the study, has been approved by The Regional Committee for Medical Research Ethics, Western Norway.

3. Results

Full scan GC–MS analysis of the tailor-made internal standard showed one peak only, which was well separated from the peaks of HEMA and TEGDMA respectively and no traces of HEMA or TEGDMA were seen. Chromatograms for scan mode and for single ion monitoring mode are shown in Fig. 2.

HEMA eluted from both materials (Fig. 3). The mean value of HEMA eluted from TEC was 0.34 $\mu g\,cm^{-2}$ (R.S.D. = 3.4%), and from FZ the mean value was 0.41 $\mu g\,cm^{-2}$ (R.S.D. = 1.7%). The difference between measured amounts of HEMA from the two materials were not statistically significant ($p = 0.055$). The mean amount of TEGDMA eluted from FZ was 1.84 $\mu g\,cm^{-2}$ (R.S.D. = 1.8%). From TEC no TEGDMA was detected.

When analyzing the samples by use of full scan GC/MS, the co-initiator dimethylaminobenzoic ethyl ester (DMABEE) was also found eluting from both materials (Fig. 3), but this substance was not quantified in this study.

4. Discussion

Eluates from dental composites have been detected and quantified using several different methods, HPLC [18,19], GC/MS [20] and fluorescence spectrophotometry [21]. For low molecular weight eluates gas chromatography combined with mass spectrometry, GC/MS, is a well suited instrument [2]. Both HPLC and GC can use MS as a detector to give sensitive and selective detection. By use of GC/MS the limit of detection (LOD) and limit of quantification (LOQ) for HEMA and TEGDMA were in the range of 0.1–5 $ng\,l^{-1}$ in our study. An advantage of GC/MS compared to HPLC/MS is that comprehensive libraries are available. Such libraries make it easier to identify compounds detected in a sample. Since GC/MS analysis requires compounds that are vaporised under typical GC/MS conditions, the more polar and high molecular weight monomers Bisfenol diglycidylmetakrylate (Bis-GMA), 2,2-bis[4-(2′-methacrylyloxyethoxy)phenyl]propane (Bis-EMA) and diurethane dimethacrylate (UDMA) are better analysed by use of various HPLC-methods [4,25]. Regardless of the method used, a careful selection of internal standard must be made. With MS as a detector, isotope labelled (^2H or ^{13}C) analogues of the analyte is to be preferred. In our study, we wanted to synthesize tailor-made internal standards with similar chemical properties as the analytes, and we wanted the synthesis to be cheap and easy to perform. We therefore decided to modify HEMA and TEGDMA by reducing the double bonds with $^1H_2$ or $^2H_2$ gas (Fig. 1). By adding $^1H_2$ to the double bond of HEMA, the molecular weight (MW) will increase with two units. A disadvantage with that is that the ions of this new internal standard might interfere with the ^{18}O containing ions

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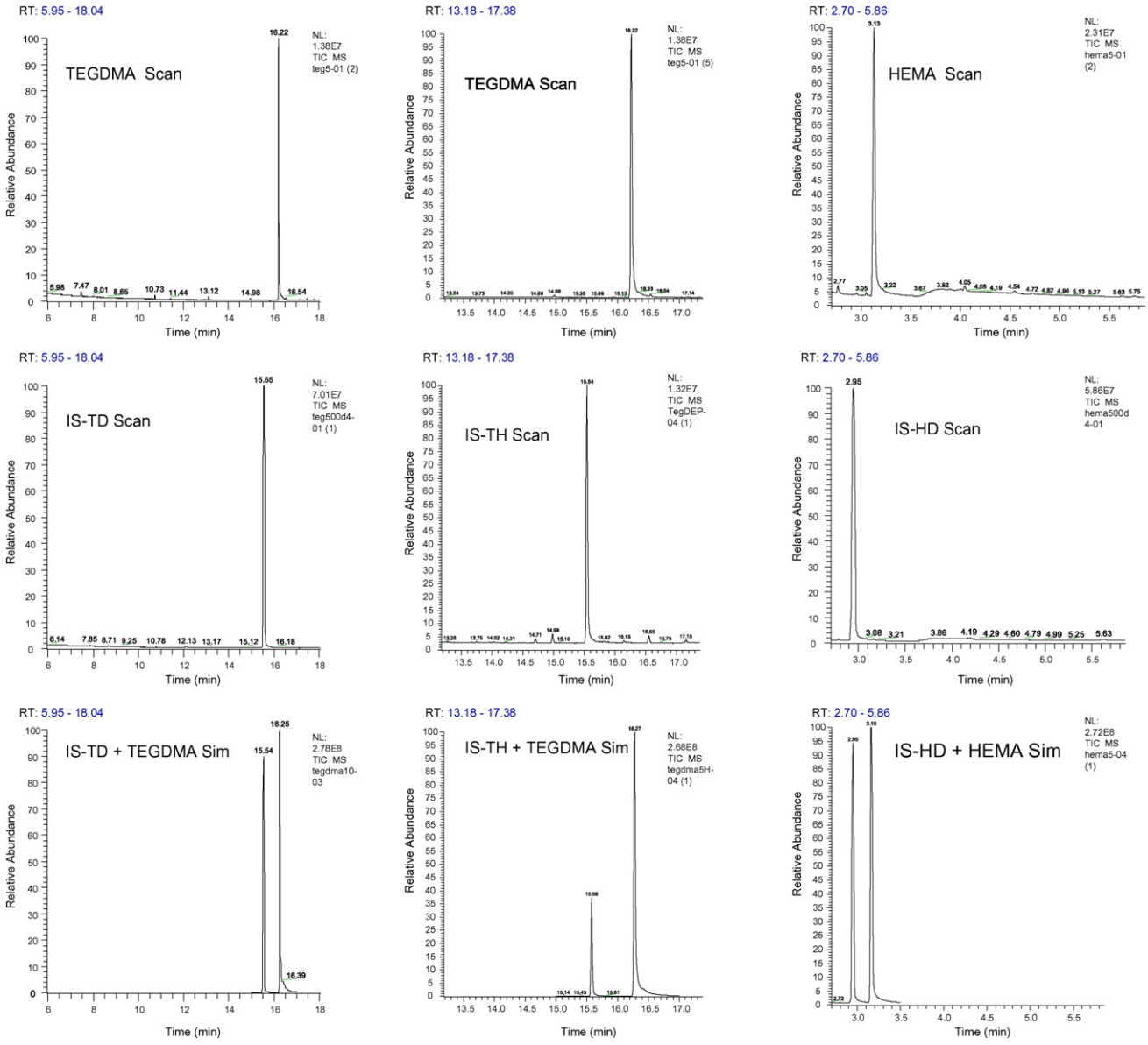

Fig. 2 – TEGDMA in scan mode, IS-TD in scan mode, IS-TD and TEGDMA in SIM mode. TEGDMA in scan mode, IS-TH in scan mode, IS-TH and TEGDMA in SIM mode. HEMA in scan mode, IS-HD in scan mode, IS-HD and HEMA in SIM mode.

of HEMA. This problem is avoided if $^{2}H_2$ are added to the double bond, increasing the MW with 4 mass units. Since TEGDMA contains two methacrylic acid double bonds, an addition of $^{1}H_2$ to these double bonds will increase the MW with 4 mass units, which will make such an internal standard suitable for analysis of TEGDMA, but only if TEGDMA give a high yield of the molecular ion. Since the yield of molecular ions are low when analysing HEMA and TEGDMA with GC/MS, internal standards made by adding $^{2}H_2$ to the methacrylic acid double bonds are to be preferred in both cases. For practical reasons the synthesized internal standards were labelled IS-TD, IS-TH and IS-HD, according to Table 1.

In quantification studies of eluates from dental materials, external standards are common, but have its limitations due to inaccuracy. Internal standard has been used only in a few studies concerning elution from the dental composites [4,17,22–24]. Caffeine has been applied as internal standard [4,24], but is not useful for saliva samples because of high contamination probability. In contrast to caffeine and other standards previously used, like diethyl phthalate [4,17,24], the synthesized internal standards elute very close to the analyte peaks, but are still well separated from the corresponding analytes (Fig. 2). Fig. 3 illustrates that diethyl phthalate is eluted far from TEGDMA and HEMA. Another advantage with the tailor-made standards is that they in all steps of the analytical procedure have properties similar to the actual analyte.

The tailor-made internal standards IS-HD, and IS-TD and IS-TH were synthesized using two strategies; adding of a proton (^{1}H) or deuterium (^{2}H) at the methacrylic double bonds in the molecules (Fig. 1). The products are new substances which will have similar properties as the analytes, but are easily separated in the GC–MS analysis. The compounds synthesized

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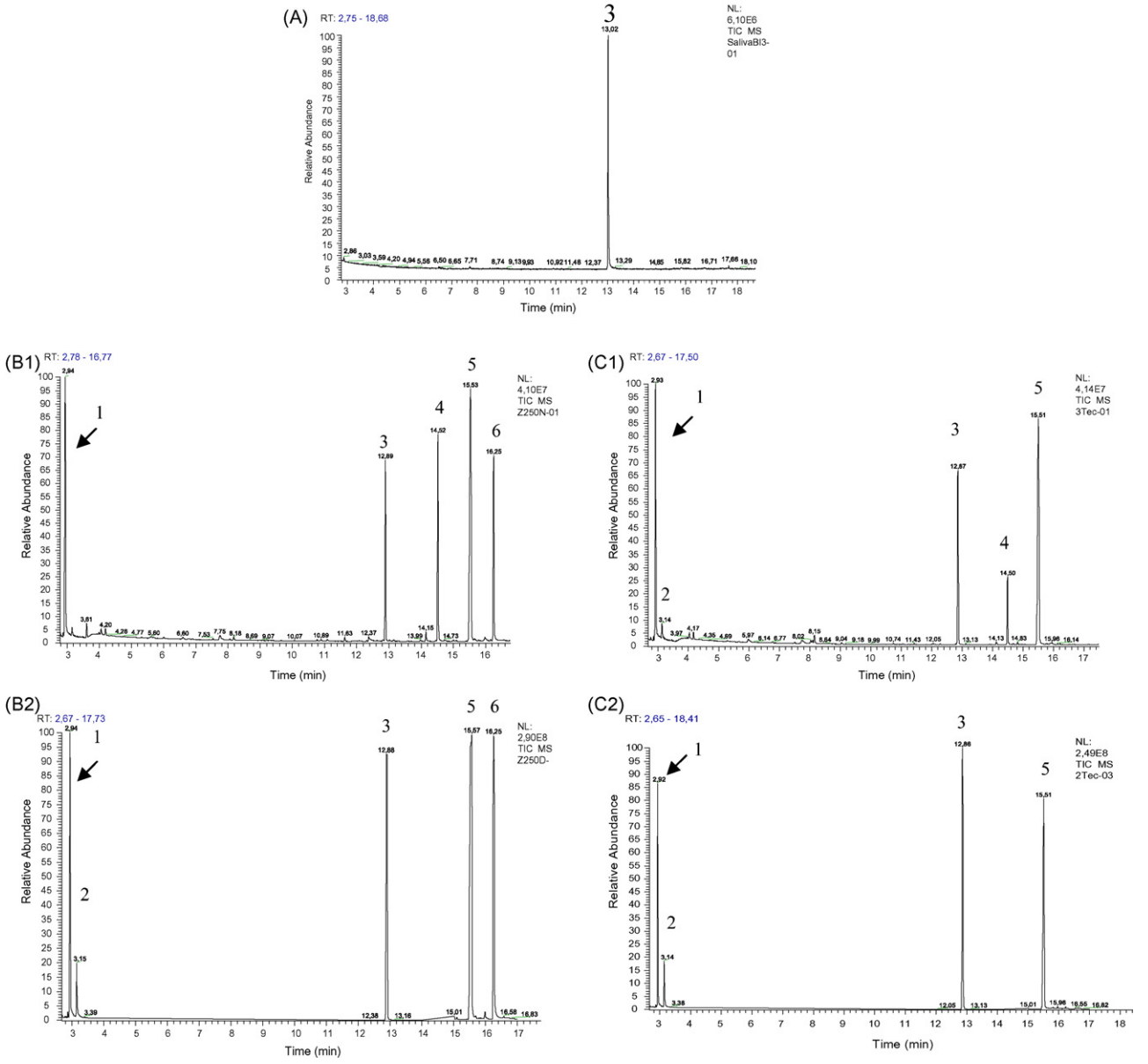

Fig. 3 – Chromatograms of: (A) human saliva with internal standard of diethyl phthalate, 2 μg ml^{-1}, scan mode. (B1) Eluted substances from FZ in scan mode. (B2) Eluted substances from FZ in SIM mode. (C1) Eluted substances from TEC in scan mode. (C2) Eluted substances from TEC in SIM mode. 1: IS-HD, 2: HEMA, 3: DEP, 4: dimethylaminobenzoic ethyl ester, 5: IS-TD, 6: TEGDMA.

were analyzed with GC/MS. Full scan for IS-TD revealed one peak only, which had a RT separate from RT of TEGDMA (Fig. 2). Mass spectra confirmed that TEGDMA had been deuterated with molecule ions and base peak ions according to Table 3. The same results apply for IS-TH and IS-HD.

Instrumental and method performance was evaluated by analyzing standards, spiked samples and blanks (Figs. 2 and 3). For verification of selectivity, the identification of the different compounds was confirmed by match of the retention time and mass spectrum against the reference substance. The analytes and the internal standards (IS-TD, IS-TH and IS-HD) gave low abundant molecule ions only. For each analyte and each IS we used the base peak ion for quantifications and also one characteristic ion for each compound as a qualifier ion to exclude the influence of possible unknown co-eluting compounds (Table 3).

The linearity, which expresses the correlation between the area of a peak and the amount of the substance, is an important method validation parameter. For both substances the linearity was good in our selected concentration range ($R^2 = 0.9942$ for HEMA and $R^2 = 0.9978$ for TEGDMA), and the concentrations of eluted substances calculated from the samples were inside the linearity range.

Confidence to an analytical result is expressed in precision tests. Within-day precision was between R.S.D. 0.03 and 2.5% ($n = 10$) for the reference substances, and intraday variation R.S.D. calculated during 2 days, was between 0.12 and 1.56% ($n = 20$). Linearity, precision and accuracy were satisfy-

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ing between 0.1 and 5 $\mu g\,ml^{-1}$ and this was defined as the measuring range.

Most dental composites are based on a mixture of high molecular weight dimethacrylate monomers like Bis-GMA, Bis-EMA and/or UDMA and TEGDMA, which is a diluent and monomer conversion enhancer. No TEGDMA was detected from TEC. The monomers given in Material Safety Data Sheet (MSDS) from TEC are Bis-GMA and UDMA. TEC is a composite with fillers in the nano scale size, whereas FZ is a hybrid composite. A high filler to resin content is desired for a high performance composite. Traditionally the viscosity increases with higher filler content, but incorporating nano-scaled fillers in the composites does not increase the viscosity to the same extent as the traditional fillers do with the same ratio of filler to resin, thus the presence of nano particles reduces the need for plasticizers. This may be the reason why the common plasticizer TEGDMA is not found eluting from TEC.

Small amounts of HEMA were detected from both materials. If this was due to evaporation and thereby low recovery of HEMA, one would expect low recovery of IS-HD also. This is not the case. HEMA is not given as an ingredient from the producers of the composites, but several studies have confirmed the presence in TEC and FZ [4,17,25]. HEMA may be a degradation product from UDMA [4,17,25] which is an ingredient in both TEC and FZ, according to MSDS.

For practical reasons saliva had to be frozen after collection and thawed before immersion of the samples. Effect of freezing and thawing of saliva containing reference substances were tested in pilot studies by analyzing different quantities of reference substances added to fresh saliva, compared to the same amounts added to fresh saliva which had been frozen and thawed. No interference was found after freezing. In our study the eluates from the saliva solution were extracted with ethyl acetate three times. Previously we have confirmed that eluates were present only in the first and in the second extraction [17]. The recovery test for HEMA in concentrations 10 and 1 $\mu g\,ml^{-1}$ was 75 and 104%, and for TEGDMA in concentrations 10 and 1 $\mu g\,ml^{-1}$ the recovery was 94 and 78%, respectively [3]. Recovery from saliva compared to recovery from water was 98.5% for HEMA and 87% for TEGDMA. During extraction internal standards and corresponding analytes' ratios are expected to be constant, thus the internal standards were added before extraction. Even though evaporation of the ethyl acetate was performed at 53 °C, which is far below the boiling point of HEMA (198 °C according to supplier Rohm and Hass Company, Philadelphia, USA), a loss of HEMA and IS-HD was detected. If the ethyl acetate solution was evaporated to less than 50% of its original volume, the loss increased gradually, however, the ratio between HEMA and IS-HD remained constant. When samples were evaporated to complete dryness, HEMA and IS-HD disappeared. The robustness is therefore considered limited concerning evaporation, and care must be taken at the evaporation process.

The retention times for the internal standards, IS-TD, IS-TH and IS-HD were lower than for the corresponding analytes, although the molecular weights are higher after reduction with $^1H_2$ and $^2H_2$. We believe this is due to an interaction between the double bond of the methacrylate group and the stationary phase of the column. The hydrogenated IS-TH was eluted before IS-TD and TEGDMA, confirming this assumption.

During synthesis of the internal standards the methacrylate part is reduced. Our applied method may therefore be used to other methacrylate monomers to produce internal standards. Due to high cost of deuterium, ^1H (hydrogenated) standards may have an advantage over the deuterated analogue. Further studies will address the *in vivo* situation by collecting samples from patients who receive resin-based restorative fillings, and identify and quantify the eluates by use of the described method.

REFERENCES

[1] Al-Hiyasat AS, Darmani H, Milhem MM. Cytotoxicity evaluation of dental resin composites and their flowable derivatives. Clin Oral Investig 2005;9(1):21–5.
[2] Geurtsen W. Substances released from dental resin composites and glass ionomer cements. Eur J Oral Sci 1998;106(2 Pt 2):687–95.
[3] Michelsen VB, Lygre H, Skålevik R, Tveit AB, Solheim E. Identification of organic eluates from four polymer-based dental filling materials. Eur J Oral Sci 2003;111(3):263–71.
[4] Spahl W, Budzikiewicz H, Geurtsen W. Determination of leachable components from four commercial dental composites by gas and liquid chromatography/mass spectrometry. J Dent 1998;26(2):137–45.
[5] Geurtsen W, Lehmann F, Spahl W, Leyhausen G. Cytotoxicity of 35 dental resin composite monomers/additives in permanent 3T3 and three human primary fibroblast cultures. J Biomed Mater Res 1998;41(3):474–80.
[6] Hanks CT, Strawn SE, Wataha JC, Craig RG. Cytotoxic effects of resin components on cultured mammalian fibroblasts. J Dent Res 1991;70(11):1450–5.
[7] Kanerva L, Estlander T, Jolanki R. Allergic contact dermatitis from dental composite resins due to aromatic epoxy acrylates and aliphatic acrylates. Contact Dermatitis 1989;20(3):201–11.
[8] Schweikl H, Altmannberger I, Hanser N, Hiller KA, Bolay C, Brockhoff G, et al. The effect of triethylene glycol dimethacrylate on the cell cycle of mammalian cells. Biomaterials 2005;26(19):4111–8.
[9] Samuelsen JT, Dahl JE, Karlsson S, Morisbak E, Becher R. Apoptosis induced by the monomers HEMA and TEGDMA involves formation of ROS and differential activation of the MAP-kinases p38, JNK and ERK. Dent Mater 2007;23(1):34–9.
[10] Spagnuolo G, Galler K, Schmalz G, Cosentino C, Rengo S, Schweikl H. Inhibition of phosphatidylinositol 3-kinase amplifies TEGDMA-induced apoptosis in primary human pulp cells. J Dent Res 2004;83(9):703–7.
[11] Paranjpe A, Bordador LC, Wang MY, Hume WR, Jewett A. Resin monomer 2-hydroxyethyl methacrylate (HEMA) is a potent inducer of apoptotic cell death in human and mouse cells. J Dent Res 2005;84(2):172–7.
[12] Stanislawski L, Lefeuvre M, Bourd K, Soheili-Majd E, Goldberg M, Perianin A. TEGDMA-induced toxicity in human fibroblasts is associated with early and drastic glutathione depletion with subsequent production of oxygen reactive species. J Biomed Mater Res A 2003;66(3):476–82.
[13] Lefeuvre M, Bourd K, Loriot MA, Goldberg M, Beaune P, Perianin A, et al. TEGDMA modulates glutathione transferase P1 activity in gingival fibroblasts. J Dent Res 2004;83(12):914–9.
[14] Lefeuvre M, Amjaad W, Goldberg M, Stanislawski L. TEGDMA induces mitochondrial damage and oxidative stress in human gingival fibroblasts. Biomaterials 2005;26(25):5130–7.

Please cite this article in press as: Michelsen VB, et al., Quantitative analysis of TEGDMA and HEMA eluted into saliva from two dental composites by use of GC/MS and tailor-made internal standards, Dental Mater (2007), doi:10.1016/j.dental.2007.08.002

[15] Noda M, Wataha JC, Lewis JB, Kaga M, Lockwood PE, Messer RL, et al. Dental adhesive compounds alter glutathione levels but not glutathione redox balance in human THP-1 monocytic cells. J Biomed Mater Res B Appl Biomater 2005;73(2):308–14.
[16] Darmani H, Al-Hiyasat AS. The resin monomer triethylene glycol dimethacrylate exhibits reproductive toxicity in male mice. Reprod Fertil Dev 2005;17(4):401–6.
[17] Michelsen VB, Moe G, Skålevik R, Jensen E, Lygre H. Quantification of organic eluates from polymerized resin-based dental restorative materials by use of GC/MS. J Chromatogr B Analyt Technol Biomed Life Sci 2007;850(1–2):83–91.
[18] Örtengren U, Wellendorf H, Karlsson S, Ruyter IE. Water sorption and solubility of dental composites and identification of monomers released in an aqueous environment. J Oral Rehabil 2001;28(12):1106–15.
[19] Hamid A, Hume WR. A study of component release from resin pit and fissure sealants in vitro. Dent Mater 1997;13(2):98–102.
[20] Geurtsen W, Spahl W, Leyhausen G. Variability of cytotoxicity and leaching of substances from four light-curing pit and fissure sealants. J Biomed Mater Res 1999;44(1):73–7.
[21] Örtengren U, Langer S, Göransson A, Lundgren T. Influence of pH and time on organic substance release from a model dental composite: a fluorescence spectrophotometry and gas chromatography/mass spectrometry analysis. Eur J Oral Sci 2004;112(6):530–7.
[22] Mazzaoui SA, Burrow MF, Tyas MJ, Rooney FR, Capon RJ. Long-term quantification of the release of monomers from dental resin composites and a resin-modified glass ionomer cement. J Biomed Mater Res 2002;63(3):299–305.
[23] Müller H, Olsson S, Söderholm K-J. The effect of comonomer composition, silane heating, and filler type on aqueous TEGDMA leachability in model resin composites. Eur J Oral Sci 1997;105(4):362–8.
[24] Geurtsen W, Spahl W, Leyhausen G. Residual monomer/additive release and variability in cytotoxicity of light-curing glass-ionomer cements and compomers. J Dent Res 1998;77(12):2012–9.
[25] Spahl W, Budzikiewicz H. Qualitative analysis of dental resin composites by gas and liquid chromatography/mass spectrometry. Fresenius J Anal Chem 1994;350:684–91.

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