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Inorganic elemental analysis and identification of residual monomers released from different glass ionomer cements in cell culture medium

Análise elementar inorgânica e identificação de monômeros residuais orgânicos liberados por diferentes cimentos de ionômero de vidro

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Resumo

Introdução: Os cimentos de ionômero de vidro (CIVs) liberam elementos inorgânicos e monômeros orgânicos residuais que têm o potencial de causar efeitos deletérios sobre as células pulpares. **Objetivo:** Identificar e quantificar os elementos inorgânicos presentes em diferentes CIVs, bem como os componentes liberados por estes materiais em meio de cultura celular. **Material e método:** Espécimes cilindricos de dois CIVs modificados por resina para base/forramento (Vitrebond e Fuji Lining LC), dois CIVs modificados por resina restauradores (Vitremer e Fuji II LC) e dois CIVs convencionais restauradores (Ketac Fil Plus e Ketac Molar Easymix) foram preparados e analisados por Espectrometria de Fluorescência de Raios X por Energia Dispersiva (EDXRF). Em seguida, extratos de 24h desses materiais foram obtidos e analisados por EDXRF e por Cromatografia Gasosa/Espectrometria de Massa (CG/EM). **Resultado:** Os elementos inorgânicos identificados em maior porcentagem nos CIVs Vitrebond, Fuji Lining LC, Vitremer, Fuji II LC e Ketac Fil Plus foram estrôncio, silício e alumínio, enquanto o zinco foi detectado apenas no Vitrebond. O Ketac Molar Easymix apresentou maior porcentagem dos elementos lantânio, cálcio, alumínio e silício. Estrôncio foi detectado nos extratos de todos os materiais, exceto no Ketac Molar Easymix; cálcio estava presente no extrato do Ketac Fil Plus; zinco apenas no Vitrebond; e silício no extrato do Fuji II LC . O HEMA foi identificado nos extratos de todos os CIVs modificados por resina, e o iodobenzeno, somente no Vitrebond. **Conclusão:** Entre os CIVs estudados, o Vitrebond é o que libera mais componentes com potencial citotóxico.

Descritores: Cimentos de ionômeros de vidro; compostos inorgânicos.

Abstract

Introduction: Glass ionomer cements (GICs) release inorganic elements and organic residual monomers with the potential for deleterious effects on pulp cells. **Objective:** To identify and quantify inorganic elements present in different GICs and released components from these materials in cell culture medium. **Material and method:** Samples of two resin-modified GICs for base/liner (Vitrebond and Fuji Lining LC), two resin-modified restorative GICs (Vitremer and Fuji II LC) and two conventional restorative GICs (Ketac Fil Plus and Ketac Molar Easymix) were prepared and analyzed by Energy-Dispersive X-Ray Fluorescence Spectrometry (EDXRF). Extracts of these materials were obtained by immersion of each sample in separate containers of DMEM for 24 h (total surface-liquid ratio = 45.7 mm^2/mL). The extracts were analyzed by EDXRF and Gas Chromatography-Mass Spectrometry (GC-MS). **Result:** Higher percentages of strontium, silicon and aluminum were identified in Vitrebond, Vitremer, Fuji Lining LC, Fuji II LC, and Ketac Fil Plus, while zinc was detected only in Vitrebond. Ketac Molar Easymix presented a greater atomic composition of lanthanum, calcium, aluminum and silicon. Strontium was detected in the extracts from all materials except Ketac Molar Easymix; calcium was present in extracts from Ketac Fil Plus; zinc only in Vitrebond; and silicon in Fuji II LC extract. The analysis by GC-MS detected 2-hydroxyethyl-methacrylate (HEMA) in the extracts from all resin-modified GICs, and iodine benzene was detected only in the Vitrebond extract. **Conclusion:** Of the GICs sampled, Vitrebond released the highest number of components with cytotoxic potential.

Descriptors: Glass ionomer cements; inorganic chemicals.

INTRODUCTION

Due to their anticariogenic properties, glass ionomer cements (GICs) play an important role in preventing secondary caries, which is the most frequent cause for replacement of restorations[1,2]. Based on their chemical compositions, these materials can be classified as (1) chemically cured GICs and (2) resin-modified GICs. Chemically cured GICs consist of a powder made up of glass particles (calcium aluminum fluoride silicate glass) and a liquid made up of polyacrylic acids[3], and their setting reaction depends on the acid reaction with the glass particle surface after mixing (acid-base reaction). Resin-modified GICs incorporate organic resin monomers like 2-hydroxyethyl methacrylate (HEMA) and photosensitive polymerization initiators in addition to glass particles and polyacrylic acids[4]. In these materials, light activation causes an initial set that is followed by an acid-base setting reaction.

The amounts of fluoride released by GICs indicate their anticariogenic potential[5-7]. However, several other inorganic elements or ions are released from GICs, and the type and amount of these depend both on the chemical composition of the glass used to manufacture the cement powder and the local pH conditions, with greater release occurring under acidic conditions[8-10].

In resin-modified GICs, organic residual monomers are also released, along with products of degradation of the photoinitiators. Together with the release of ions and/or inorganic elements, these constitute important factors that can influence the cytotoxicity of these materials[11-16].

Since the ions, residual monomers, and other components released from GICs have the potential for deleterious effects on pulp cells, it is important to evaluate the release of these components in cell culture medium with a pH near neutral before assessing their in vitro and in vivo cytotoxic effects. Thus, this study aimed to identify the inorganic elements present in different GICs as well as to evaluate inorganic elements and organic residual monomers present in the extracts from these materials. The null hypothesis advanced was that the lixiviated components from the GIC are not dependent on the type of GIC.

MATERIAL AND METHOD

The commercially available glass ionomer cements used in this study as well as the main composition of the products, powder/liquid ratio by weight, and curing times are listed in Table 1. The experiments were carried out at 24 ± 1 °C room temperature. Moreover, each measurement was repeated three times to ensure reliable results.

1. *Identification and Analysis of Inorganic Elements Present in Glass Ionomer Cements*

The study materials were handled at room temperature (24 ± 1 °C), according to the respective manufacturers' instructions, and placed with the aid of a Centrix syringe (DFL Indústria e Comércio SA, Rio de Janeiro, Brazil) in a stainless steel matrix measuring 10 mm in diameter and 1 mm thick. A plastic matrix strip and glass slide were placed on the surface of the material and pressed with a weight of 500 gf to promote the overflow of the material. Resin-modified GICs were light-activated using an halogen light unit (Optilux 500, Kerr Company, Orange, USA) positioned about one millimeter from the surface of the specimen. The light intensity was monitored with a radiometer (average 450 ± 10 mW/cm^2). Conventional GICs remained in the matrix for 10 minutes in the presence of ambient light to ensure the initial setting.

The specimens were removed from the matrix and placed in an incubator with 100% humidity at 37 °C for 60 minutes. After this period, the specimens were analyzed by Energy-Dispersive X-Ray Fluorescence Spectrometry (EDX800, Shimadzu, Tokyo, Japan) for simultaneous quantitative determination of atomic inorganic multi-elements (at. %) ranging from Sodium (Na) to Uranium (U). Before the analysis, the spectrometer was calibrated to a standard aluminum sample. The X-ray generator with a rhodium (Rh) tube was operated at a tube voltage of 50 kV, tube current of 20 mA, and collimator size of 10 mm diameter without primary filters and with an air cooling method. The sample chamber was conditioned to a vacuum atmosphere and the detector was cooled to –174 °C by the LN_2 method (liquid nitrogen). Three specimens of each material were analyzed under these conditions.

Next, the specimens were immersed in DMEM cell culture medium (Sigma Chemical CO., St. Louis, MO, USA) without fetal calf serum (total surface-liquid ratio = 45.7 mm^2/mL) for 24 hours in separate containers. Finally, they were rinsed with distilled water and analyzed by EDXRF again.

2. *Identification and Analysis of Inorganic Elements Present in Extracts from the Glass Ionomer Cements*

Three specimens of each material were placed in individual compartments of sterile acrylic plates with 12 wells (Costar Corp., Cambridge, MA, USA) containing 4.1 mL of DMEM culture medium (Sigma Chemical Co., St. Louis , MO, USA) without fetal calf serum (total surface-liquid ratio = 45.7 mm^2/mL). The specimens were incubated for 24 hours at 100% humidity and 37 °C to obtain the extracts from the materials. The extracts were then analyzed by Energy-Dispersive X-Ray Fluorescence Spectrometry (EDX800, Shimadzu, Tokyo, Japan) for determination of atomic inorganic multi-elements (at. %). Before the analyses, the spectrometer was calibrated to a standard aluminum sample. The X-ray generator with a rhodium (Rh) tube was operated at a tube voltage of 50 kV, tube current of 20 mA, and a collimator size of 10 mm diameter without primary filters and with an air cooling method. The sample chamber was conditioned to air atmosphere, and the detector was cooled to –174 °C by the LN_2 method (liquid nitrogen). The DMEM pure culture medium was used as a negative control of the experiment.

3. *Identification of 2-Hydroxyethyl-methacrylate (HEMA) and Iodine Benzene in the Extracts from Glass Ionomer Cements*

We prepared six specimens measuring 4 mm in diameter and 2 mm thick for each experimental group. Each sample was placed in 1.1 mL of DMEM culture medium without fetal calf

Table 1. Commercially available glass ionomer cements used in the study

GICs (manufacturer)	Composition (% weight)	Classification : Indication[17]	Powder/ Liquid ratio	MRT or initial curing time
Vitrebond (3M, ESPE Dental Products, St. Paul, MN, EUA)	*Powder* glass powder (>95%) diphenyliodonium chloride (<2%)	Resin-modified GIC : lining/base	1.4/1	30 s
	Liquid copolymer of acrylic and itaconic acids (35-45%) 2-hydroxyethyl methacrylate - HEMA (20-30%) water (30-40%)			
Vitremer (3M, ESPE Dental Products, St. Paul, MN, EUA)	*Powder* *silane treated glass* (90-100%) potassium persulfate < 1 %	Resin-modified GIC : restoration	2.5/1	40 s
	Liquid copolymer of acrylic and itaconic acids (45-50%) 2-hydroxyethyl methacrylate - HEMA (15-20%) water (25-30%)			
Fuji Lining LC (GC, Tokyo, Japão)	*Powder* alumino-silicate glass (100%)	Resin-modified GIC : lining/base	1.4/1	30 s
	Liquid polyacrylic acid (65-70%) 2-hydroxyethyl methacrylate, HEMA (8-10%) Proprietary ingredient (5-15%)			
Fuji II LC (GC,Tokyo, Japão)	*Powder* alumino-silicate glass –100%	Resin-modified GIC : restoration	3.0/1	40 s
	Liquid polyacrylic acid (20-22%) 2-hydroxyethyl methacrylate, HEMA (35-40%) proprietary ingredient (5-15%) 2,2,4 trimethyl hexamethylene dicarbonate (5-7%) triethylene glycol dimethacrylate (4-6%)			
Ketac Fil Plus (3M, ESPE Dental Products, St. Paul, MN, EUA)	*Powder* glass powder (≈100%)	Convencional GIC : restoration	3.2/1	7 min
	Liquid water (60-65%) polyethylene polycarbonic acid (30-40%) tartaric acid (5-10%)			
Ketac Molar Easymix (3M, ESPE Dental Products, St. Paul, MN, EUA)	*Powder* glass powder (85-95%) polyacrylic acid - 5-15%	Convencional GIC : restoration	2.9/1	5 min
	Liquid water (55-65%) polyethylene polycarbonic acid (25-35%) tartaric acid (5-10%)			

*MRT=manufacturer recommended curing time

serum for 24 hours in an incubator with 100% humidity at 37 °C to obtain the extracts (total surface-liquid ratio = 45.7 mm^2/mL). These extracts were analyzed by Gas Chromatography-Mass Spectrometry (GC-MS) to identify the organic residual monomers released from GICs into the DMEM culture medium.

Analyses were conducted with a Shimadzu GC-MS automated gas chromatograph-mass spectrometer model GC-17A/QP-5050A coupled to a model AOC20i auto-sampler. It was manipulated with GCMS Solutions v.1.02 workstation software (Shimadzu, Kyoto, Japan). All analyses were obtained using EI/MS, positive ion mode, full-scan acquisition mode (40 to 500 m/z), and "hard" energy ionization (70 eV).

We performed chromatographic separation on a fused-silica capillary nonpolar column SPB™–1 (30 m × 0.25 mm i.d. × 0.10 µm film thickness; Supelco Inc., Bellefonte, PA, USA) with dimethylpolysiloxane as the stationary phase and helium as carrier gas at a constant flow (1 mL min^{-1}). Sample aliquots (1 µL) were injected in split mode (1:20) with solvent cut time (3 min). The injector and interface detector temperatures were maintained at 260 °C and 280 °C, respectively. The oven temperature was

initially kept at 60 °C for 2 min then increased to 180 °C at 2 °C min^{-1}. Finally, the temperature was raised to 240 °C at 10 °C min^{-1}, and kept constant for 10 min, completing 78 min of total analysis.

Residual organic monomers 2-hydroxyethyl methacrylate (HEMA) and iodine benzene (IB) were identified by comparing retention times and mass spectra of chromatograms of the extracts from GICs with chromatograms of authentic standard HEMA (Sigma Chemical Co., St. Louis, USA) and iodine benzene (Sigma Chemical Co., St. Louis, USA) injected under the same conditions.

RESULT

1. *Identification and Analysis of Inorganic Elements Present in Glass Ionomer Cements*

The quantitative atomic inorganic multi-elemental composition (at.%) of the glass ionomer cements Vitrebond (VB), Vitremer (VT), Fuji Lining LC (FL), Fuji II LC (FII), Ketac Fil Plus (KP), and Ketac Molar Easymix (KM) determined by EDXRF are listed in Table 2.

Figure 1 shows EDXRF wide scans up to 40 keV binding energy for the glass ionomer cements. All glass ionomer cement samples exhibit significant signals of their main components with varying intensities quantified in Table 2. Moreover, as can be seen in Figure 1, all samples have very similar spectral profiles, except for Ketac Molar Easymix (KM).

The quantitative atomic inorganic multi-elementals (at.%) identified at higher levels in the VB, VT, FL, FII and KP glass ionomer cements were strontium (Sr), silicon (Si) and aluminum (Al), while zinc (Zn) was only detected in VB. On the other hand, KM presented lanthanum (La), calcium (Ca), aluminum (Al), and silicon (Si) as its main atomic inorganic elements, and strontium (Sr) was not detected in this material. Moreover, after 24 hours in contact with DMEM culture medium, the inorganic multi-elemental composition was unchanged in all glass ionomer cements. Only a small decrease or increase of main elements could be seen.

2. *Identification and Analysis of Inorganic Elements Present in DMEM Extracts from Glass Ionomer Cements*

The quantitative atomic inorganic multi-elemental compositions (at.%) of all GICs extracts detected by EDXRF are listed in Table 3. Pure DMEM was used as an internal negative control. Figure 2 shows EDXRF wide scans up to 40 keV binding energy for extracts from all glass ionomer cements. As can be seen in Figure 2, all samples have very similar spectral profiles except for Ketac Molar Easymix (KM). The most intensive

Table 2. Main atomic inorganic multi-elemental composition (at.%) in glass ionomer cements identified by Energy Dispersive X-Ray Fluorescence Spectrometry (EDXRF) at initial time and after 24 hours of immersion in DMEM

Group	Period	Main Atomic Inorganic Elements in GIC								
		Zn	Sr	La	Si	Al	P	Ca	Na	Fe
VB	Initial	32.9(0.4)	32.8(0.2)	-	20.8(0.4)	10.8(0.1)	1,0(0.7)	1,0(1.0)	-	1,9(1.1)
	24 hours	33.2(0.1)	33.8(0.5)	-	19.9(0.3)	9.4(0.1)	1.6(0.2)	1.4(0.1)	-	0.2(0.1)
VT	Initial	-	52.8(1.6)	-	27.5(0.2)	13.8(0.2)	2.4(0.1)	-	1.6(0.08)	0.2(0.1)
	24 hours	0.2(0,1)	54.8(2.3)	-	25.7(0.2)	11.9(0.4)	2.3(1,7)	2.4(0.3)	3.0(0.1)	0.6(0.5)
FL	Initial	0.1(0.1)	59.5(0.5)	-	23.2(0.6)	12.6(0.3)	2.6(0.04)	0.2(0.1)	-	0.9(0.9)
	24 hours	-	57.4(1.8)	-	25.5(0.4)	10.3(0.2)	3.6(0.4)	1.5(0.1)	2.1(1,2)	0.8(1.0)
FII	Initial	-	56.8(1.7)	-	29.4(1.1)	12.3(0.4)	0.9(0.3)	0.2(0.3)	-	0.2(0.01)
	24 hours	-	56.8(0.8)	-	28.8(0.3)	10.1(0.3)	2.4(0.1)	1.7(0.1)	-	-
KP	Initial	-	46.9(0.2)	7.0(0.1)	24.2(0.3)	14.5(0.3)	3.2(0.09)	0.2(0.03)	2.6(0.4)	-
	24 hours	-	47.6(1.9)	7.1(0.3)	23.0(0.5)	12.5(0.6)	3.0(0.1)	2.1(0.5)	5.0(1.7)	-
KM	Initial	-	-	30.8(2.7)	17.2(0.8)	20.0(0.7)	2.4(0.2)	26.2(1.5)	3.4(2.0)	-
	24 hours	-	-	29.5(0.2)	14.7(0.1)	16.6(0.6)	2.9(0.2)	28.5(1.0)	7.4(1.5)	-

Values represent mean (standard deviation) of three specimens. VB = Vitrebond, VT = Vitremer, FL = Fuji Lining LC, FII = Fuji II LC, KP = Ketac Fil Plus, KM = Ketac Molar Easymix

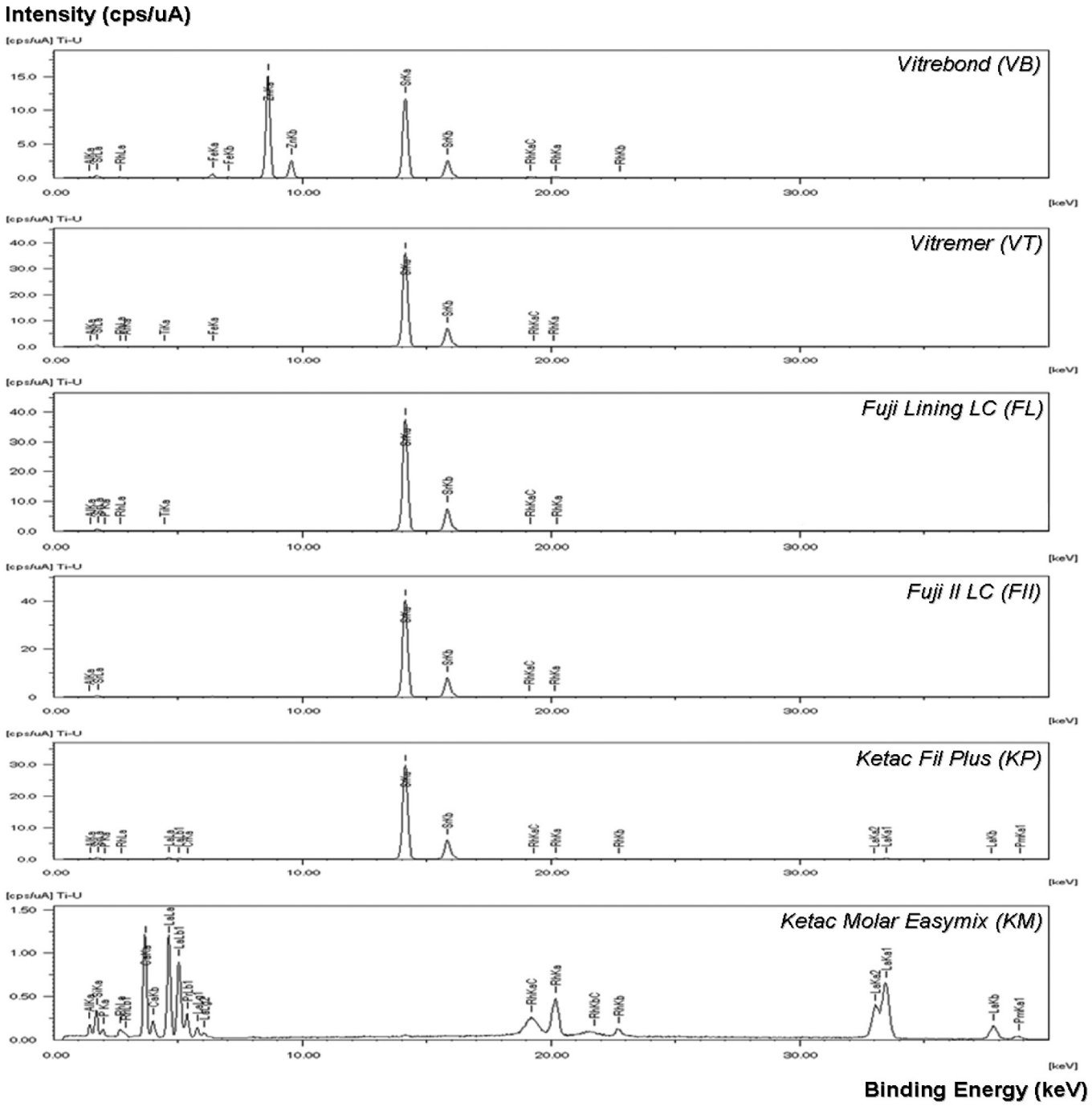

Figure 1. Spectral profile of the glass ionomer cement specimens analyzed by Energy Dispersive X-Ray Fluorescence Spectrometry (EDXRF).

Table 3. Main atomic inorganic multi-elemental composition (at.%) identified in 24-hour extracts of glass ionomer cements by Energy Dispersive X-Ray Fluorescence Spectrometry (EDXRF)

Main Inorganic Elements*	Glass Ionomer Cements Extracts						
	blank	VB	VT	FL	FII	KP	KM
Zn	-	86.5	-	-	-	-	-
Sr	-	13.5	100.0	100.0	71.2	52.1	-
Si	-	-	-	-	28.8	-	-
Ca	-	-	-	-	-	47.9	-

* mean of three samples.

signals, binding energy from 18 to 23 keV, came from the X-ray generator with a rhodium (Rh) tube. Strontium was detected in all glass ionomer cement extracts except Ketac Molar Easymix (KM). Zinc (Zn) was only identified in Vitrebond (VB) extract; silicon (Si) in Fuji II LC (FII) extract, and calcium (Ca) in Ketac Fil Plus (KP) extract.

3. *Identification of 2-Hydroxyethyl-methacrylate (HEMA) and Iodine Benzene (IB) in Extracts from Glass Ionomer Cements*

In the identification of HEMA and iodine benzene, two parameters were taken into account: retention time and

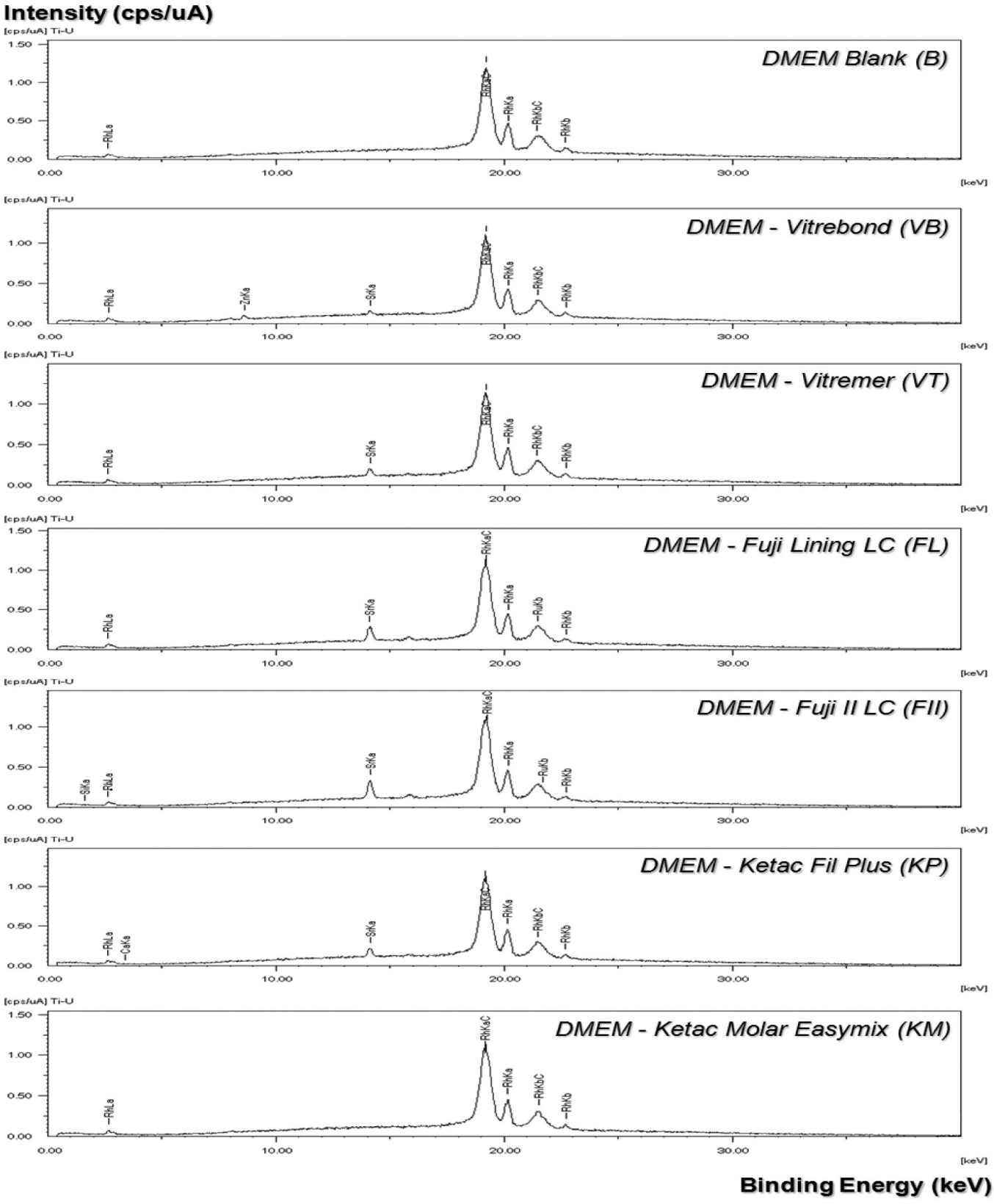

Figure 2. Spectral profile of the extracts from glass ionomer cements in cellular culture medium analyzed by Energy Dispersive X-Ray Fluorescence Spectrometry (EDXRF).

mass spectra for each of the standards injected in the same chromatographic conditions as the extracts from the cements (Figure 3).

The chromatographic profiles obtained by GC/MS of DMEM culture medium containing organic residual monomers released from resin-modified glass ionomers Vitrebond, Vitremer, Fuji Lining, and Fuji II LC, along with the chromatographic profiles of standards HEMA and iodine benzene, are illustrated in Figure 4.

HEMA was detected in all the extracts from resin-modified GICs we examined, and iodine benzene was identified only in the Vitrebond chromatogram (Figure 4). The chromatograms of extracts from resin-modified GICs showed several peaks with different retention times, suggesting the release of other monomers and/or decomposition products of initiators of polymerization, but these were not identified in this study.

DISCUSSION

The type of glass ionomer cement (conventional or resin-modified cement/liner or restorative) and the amount of released components may play an important role in the biological behavior of these materials[12,13,16]. Some ions and/or inorganic elements, such as fluoride, calcium, aluminum, silicon, strontium, and zinc, among others, may be released during the cure reaction or by solubilization of the glass ionomer cements in humid conditions[15,18-21]. However, according to Stanislawski et al.[22] (2000), the concentration of ions and/or inorganic elements released, with the exception of zinc, is insufficient to induce toxic effects in cells. In some GICs, cytotoxic effects are attributed to the release of small amounts of aluminum, iron, or copper, which can cause oxidative stress on cells in culture[23] by depletion of glutathione, generation of reactive oxygen species,[11,22] and other molecular mechanisms that can lead to apoptosis or cell death[24].

The nature of the ions and/or inorganic elements released depends on the chemical composition of the material. Therefore, in this study, before analyzing the extracts of GICs, we examined specimens of each material using Energy-Dispersive X-Ray Fluorescence Spectrometry (EDXRF). This analysis showed the percentage by mass of inorganic chemical elements present in each material (Table 2). We also analyzed extracts from the materials in cell culture medium (DMEM) using EDXRF. In the 24-hour extracts, we identified several inorganic elements (Table 3) that generally corresponded to those present in highest percentages in the materials (Table 2). We found strontium in extracts from all materials except Ketac Molar Easymix; calcium in extracts from Ketac Fil Plus; zinc only in Vitrebond; and silicon in Fuji II LC. As the methodology only allows the analysis of inorganic elements ranging from sodium (Na) to uranium (U), fluoride ion was not availed in samples. Other chemical elements were likely present at levels below those detectable by our methodology and were not identified. Differences may occur between various studies due to methodology. Forss[8] (1993) used the molybdenum blue method for detection of silicon and atomic absorption spectrometry

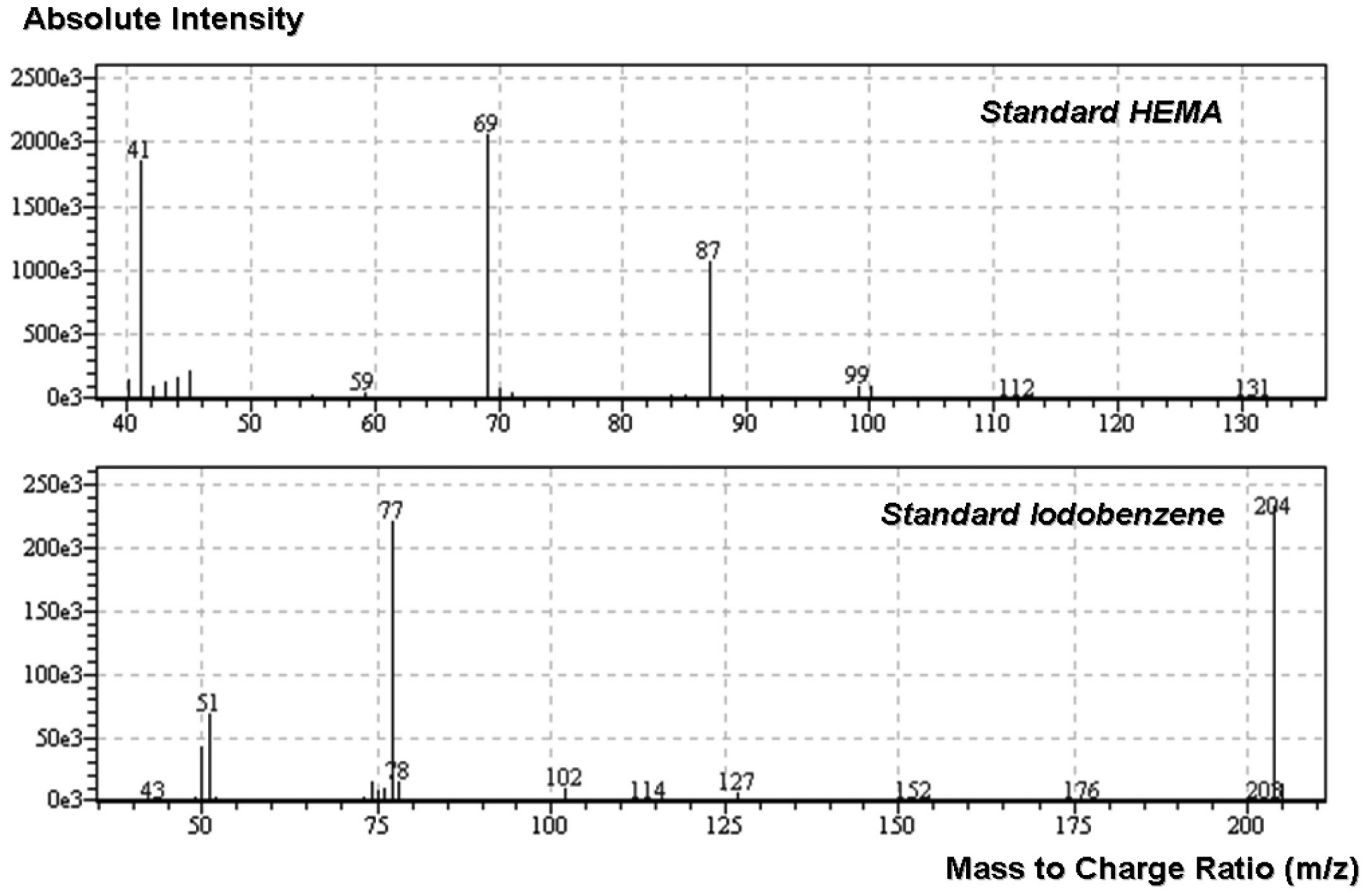

Figure 3. Mass spectra of HEMA and iodine benzene standards analyzed in the same conditions as extracts from the glass ionomer cements.

for detection of sodium, calcium, strontium and aluminum to analyze extracts from Vitrebond and Fuji Lining LC in distilled water. The author found that sodium, silicon and strontium were present in 24-hour extracts from the two materials, while aluminum was only detected in the extract from Vitrebond and calcium was not detected.

The resin-modified glass ionomers can induce varying degrees of cytotoxicity, probably due to differences in their compositions[4]. The liquid from resin-modified GICs contains resin monomers such as 2-hydroxyethyl-methacrylate (HEMA) at various concentrations. Furthermore, the cements used as liners are handled with a lower powder/liquid proportion in order to achieve better consistency and hence have higher concentrations of HEMA[25]. This monomer is easily released and diffused through the dentin due to its low molecular weight. It can incorporate into the double lipid layer of the cell membrane,

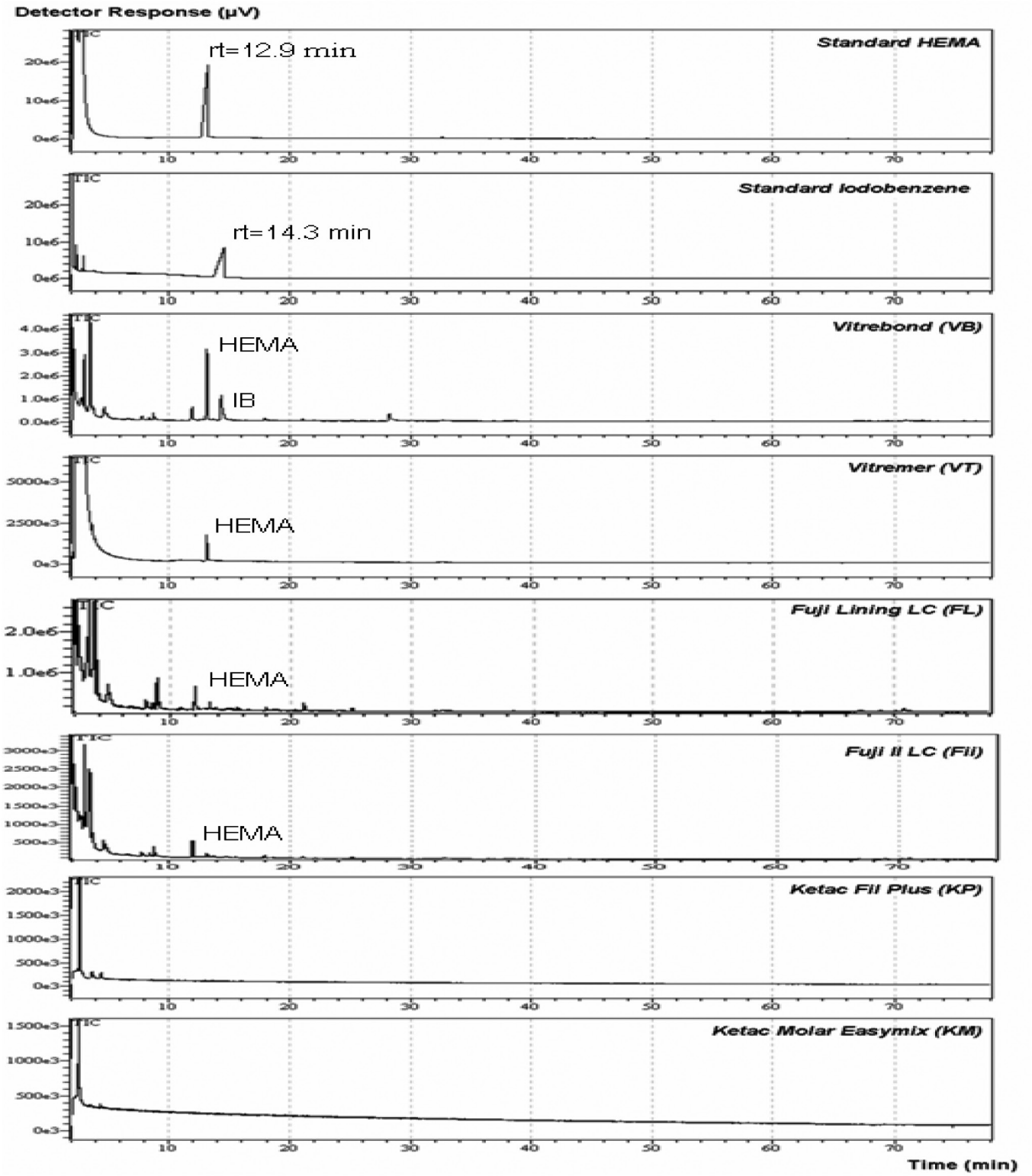

Figure 4. Authentic standard chromatograms of HEMA and iodine benzene (IB) and chromatograms of the different GIC extracts injected under the same conditions (rt = retention time in minutes).

causing its solubilization[26]. Thus, it has a highly cytotoxic effect even at low concentrations[27]. Moreover, HEMA inhibits the intracellular phosphorylation of tyrosine[28] and cell growth[29], and is associated with glutathione depletion and the production of reactive oxygen species that are determinants of apoptosis[29].

Toxic monomers that diffuse through dentin, reaching the underlying cells, are represented by residual molecules not converted into polymers during the polymerization process and molecules released by hydrolytic degradation of the polymer over time. The concentration at which these monomers reach pulp space can be considered potentially toxic, resulting in a significant reduction in cell viability and evident morphological cell changes[14,27,30,31]. In this study, since the samples were placed in the culture medium 60 minutes after its preparation and stayed in contact with it for 24 hours, the HEMA detected in GC/MS represents the percentage of unconverted monomer.

In addition to HEMA, other monomers with the potential to induce cytotoxic effects and cell apoptosis have been identified in aqueous extracts from resin materials[32,33]. while only HEMA and iodine benzene were analyzed in this study, the chromatograms of some extracts showed several peaks with different retention times, suggesting the release of other monomers and/or decomposition products of initiators of polymerization. These can be easily identified by GC/MS, according to Geurtsen et al.[11,32] (1998, 1999).

Therefore, further studies are needed to determine the quantity of all substances released and their role in the cytotoxicity of some glass ionomer cements. Furthermore, although the methodology of the present study only allowed the analysis of some released components from the GICs, it suggests that Vitrebond represents the most potentially cytotoxic material, since along with the release of HEMA, which occurs in other resin-modified GICs, this material also releases iodine benzene and zinc, a highly cytotoxic ion.

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CONFLICTS OF INTERESTS

The authors declare no conflicts of interest.

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