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Time-related bisphenol-A content and estrogenic activity in saliva samples collected in relation to placement of fissure sealants

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Abstract It was recently reported that estrogenic activity was detected in saliva samples collected during 1 h after placement of one fissure sealant (Delton) and this related to Bisphenol-A (BPA) content. The aim of the present study was to determine the time-related BPA content and estrogenic activity in saliva samples collected before and after placement of two fissure sealants each with a different monomer composition. Eight healthy male volunteers with no history of prior placement of fissure sealants or composite resin fillings had four molars sealed with either Delton LC (four people) or Visio-Seal (four people). Base-line saliva samples were collected preexperimentally, in the morning when fasting. Fissure sealants were placed and saliva samples collected immediately, 1 h and 24hs after placement of the fissure sealant. BPA was found in saliva samples collected immediately after placement of Delton LC (range 0.3–2.8 ppm). No detectable amounts of BPA were determined 1 h and 24 h after Delton treatment (detection limit ≤ 0,1 ppm). In base-line samples and in all samples collected from Visio-Seal treated individuals, no BPA was detected. In a recombinant yeast cell assay, significantly increased estrogenic activity was found in saliva samples collected immediately after placement of Delton LC sealant ($P<0.05$; ANOVA) whereas no statistically significant estrogenic activity was observed in the remaining groups. In conclusion, minute amounts of BPA, however considerably lower than previously reported, were detected in saliva samples collected immediately after but not 1 and 24 h(s) after placement of Delton LC fissure sealant. BPA was not detected after placement of Visio-Seal fissure sealant.

Key words Fissure sealants · Saliva · Bisphenol-A · Estrogenic activity · Recombinant yeast cell assay

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Introduction

In recent years the impact of certain estrogenic xenobiotics on the development, health and reproductive systems of wildlife has raised concern [3]. Accordingly the possible involvement of estrogen in falling sperm counts and disorders of the male reproductive tract has been debated [4,13]. The xenoestrogens comprise several groups of compounds that are in daily use in industry, agriculture and in the home. The major groups of environmental chemicals in question include organochlorine pesticides, polychlorinated biphenyls (PCBs), dioxins, alkylphenol polyethoxylates and phytoestrogens, as well as other xenoestrogens such as bisphenol-A (BPA) and phthalates [15]. BPA is used extensively as a plasticizer, and can be found e.g. among the epoxy resins used as plastic coatings in the food-packing industry [2]. BPA release from the lacquer coating of food cans undergoing heat sterilization has been demonstrated [2]. Polycarbonate products undergoing autoclaving were also found to release BPA during heating [6].

Xenoestrogens like BPA and phatalates may also be found in dental products: Dental (di)metacrylate-based restorative materials may thus contain xenoestrogens either as an intended ingredient (Bis-DMA) or as a residue (bisphenol-A) from the synthetization of one of the major ingredients, Bis-GMA [10,14]. Unspecific esterase and other enzymes in saliva have been shown to attack the dimethacrylate resin matrix [7]. By enzymatic hydrolysis of the ester linkage in a pendant methacrylate group, the central part of the molecule turns into an alcohol or, in the case of Bis-GMA, a divalent alcohol [10].

It has been speculated that further metabolism of the divalent alcohol in the gastrointestinal tract might lead to formation of BPA or that residual Bis-GMA may be metabolized to form BPA without prior enzymatic hydrolysis of the ester linkages [9]. One study reported release of BPA and estrogenic activity in eluates of Bis-GMA eluated under extreme conditions [9]. In a clinical part of the same study, estrogenic activity in saliva samples collected 1 h after placement of one fissure sealant was reported and related to the BPA content [9]. The results of the study caused public concern but they have since been questioned as a result of in vitro studies where previously reported findings of substances released from dental sealants could not be confirmed [5,8]). So far no further clinical studies have been reported.

The aim of the present study was to determine the time related BPA content and estrogenic activity in saliva samples collected before and after placement of two fissure sealants each with a different monomer composition.

Materials and methods

Clinical study

Each of eight healthy male volunteers (20–23 years old) with no history of prior placement of fissure sealants or composite resin fillings had four molars sealed with Visio-Seal (ESPE Dental, Seefeld, Germany; Batch no. 20536) or Delton LC pit and fissure sealant Clear (Dentsply Ash, York, PA; Batch no. 970530). Briefly, the entire surface of the molars undergoing sealant treatment was cleaned with a non-fluoride, oil free pumice paste. After thorough rinsing with a water spray the experimental teeth were isolated with cotton rolls and dried with an air syringe. Etchant was applied for 60 s. After thorough water rinsing (30 s) the teeth were dried. Areas not appearing frosty and opaque were re-etched for 20 s. Subsequently the teeth were re-isolated with fresh cotton rolls. Fissure sealant was dispensed into a pre-weighed mixing well supplied with the product kits and applied to the teeth according to the manufacturer's instructions. All coated surfaces were exposed to a visible light source (3 M Curing Light XL 1000) for 30 s keeping the light exit window 1–2 mm from the tooth surface. Subsequently, the surfaces were wiped with fresh cotton rolls.The mean amount of fissure sealant applied to each person was 38±3 mg.

Individual saliva samples (5 ml) were collected in the morning (fasting) before mouth rinsing or tooth cleaning and while the patient was still fasting to provide baseline data. The first test sample was collected immediately after placement of fissure sealant, after the recommended removal of the unpolymerized surface layer but before occlusal adjustment. Subsequent samples (5 ml) were collected 1 h and 24 h after placement of sealant. The samples were collected in 10 ml Pyrex vials holding 2500 µl ethanol (LiChrosolv; Merck, Darmstadt, Germany) (resulting saliva/ethanol dilution 2:1).They were then placed in an ultrasonic bath for 5 minutes, centrifuged at 5000 rpm for 20 minutes and filtrated through 0.2 µm filter before storage at 4 C.

Chemical analyses

The BPA content was determined by high-performance liquid chromatography (HPLC) using a C18 Hypersil ODS column (grain size: 5µm; 250×4 mm). Elution was performed with methanol/0.05 M phosphoric acid (55%/45% v/v at a flow rate of 1.3 ml/min. The injection volume was 50 µl. The elution profile was detected with a spectrofluorophotometer with an excitation wavelength of 275 nm and an emission wavelength of 300 nm. The BPA standard was obtained from Shell Netherland Chemie, Bv; Lot Nr. QC 14455433. Solvent for the standard solutions was a mixture of water/ethanol 2:1 v/v. The test solutions were consisted of a mixture of saliva/ethanol 2:1 v/v. The HPLC led to a detection limit of 0.1 ppm and a quantitation limit of 0.3 ppm.

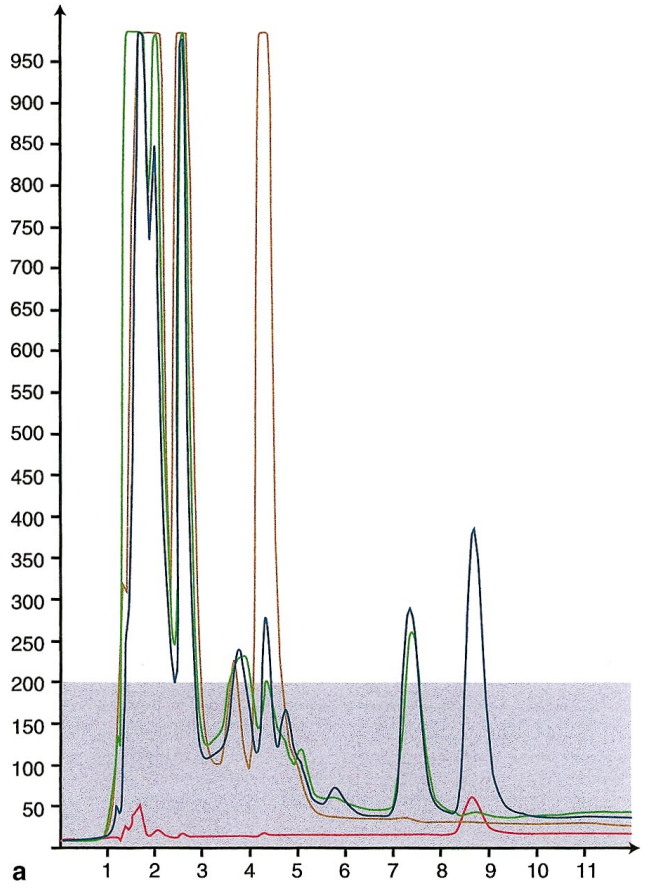
Estrogenicity assay

Estrogenic activity was assessed using an in vitro recombinant yeast cell human estrogen receptor transactivation assay [11]. Preparation of cultures, media and exposure was performed according to [1,11]. Yeast cultures were added to graded dilutions of saliva samples in 96-well multititer plates. The ethanol content of the preserved saliva samples was carefully evaporated from the vials. Fifty µl aliquots of the remaining undiluted saliva were transferred into wells containing 150 µl culture medium, thus resulting in a dilution of 1:3 (saliva/medium vol/vol, a total of 200 µl per well) in the first row. Then 25 µl undiluted saliva were added to each well in the following row (125 %), 12.5 µl to the next row (62.5% saliva) etc. Estrogenic activity was assessed spectrophotometrically by determination of β-galactosidase activity at day 4 of exposure. Absorbance readings were performed at 540/600 nm. Graded dilutions of BPA (downward from 50 ppm) served as positive controls.

Results

BPA content in saliva samples is shown in Table 1. Samples in all but one group had BPA levels equal to or below the detection limit of 0.1 ppm. In samples collected immediately after placement of Delton LC, BPA levels varied within the range of 0.3–2.8 ppm (mean 1.43 ppm) whereas no BPA was detected in samples collected immediately after placement of Visio-Seal fissure sealant.

Typical chromatograms showing HPLC analyses of saliva samples from two patients receiving Delton or Visio-Seal respectively are shown in Figs. 1a-b and 2. Chromatograms from saliva samples collected from a patient immediately after placement of Delton fissure



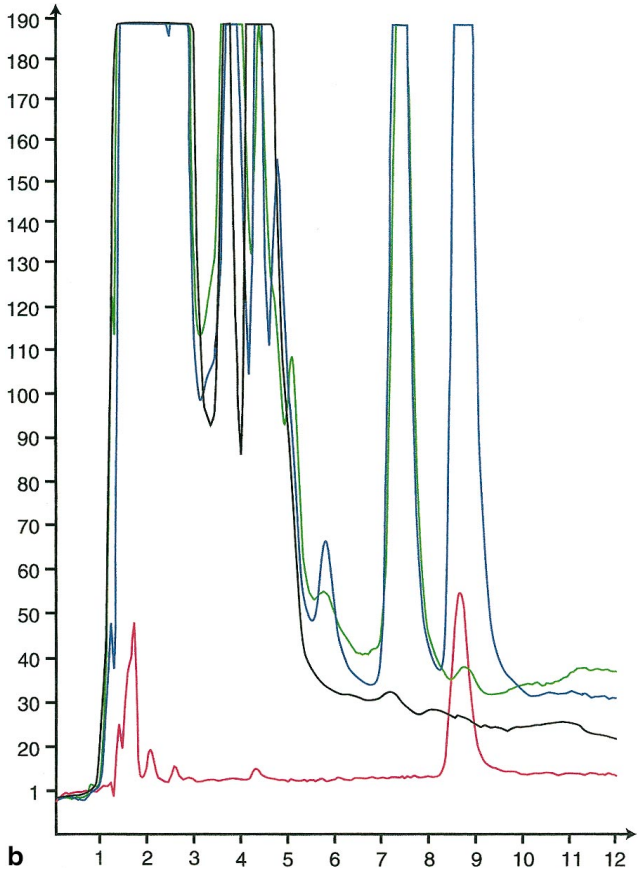


Fig. 1 a Chromatogram showing the elution profile of saliva samples collected before and after placement of Delton LC fissure sealant (patient G). Baseline (brown); Immediately after fissure sealant application (blue); 1 h after fissure sealant placement (green); BPA standard, 66 ppb (red). **b** Extended section of the elution profile shown in Fig. 1a (scattered section)

Table 1 BPA content in saliva samples collected before (sample 1) and after (sample 2: immediately after; sample 3: 1 h after; sample 4: 24 h after) placement of fissure sealant (mean 38±3 mg per person). Detection limit 0.1 ppm. Quantification limit 0.3 ppm. D: Delton LC, VS: Visio-Seal.

Patient	Bisphenol-A concentration (ppm)			
	Sample 1	Sample 2	Sample 3	Sample 4
A (VS)	≤0,1	≤0,1	≤0,1	≤0,1
B (VS)	≤0,1	≤0,1	≤0,1	≤0,1
D (VS)	≤0,1	≤0,1	≤0,1	≤0,1
H (VS)	≤0,1	≤0,1	≤0,1	≤0,1
C (D)	≤0,1	1,8	≤0,1	≤0,1
E (D)	≤0,1	≤0,3	≤0,1	≤0,1
F (D)	≤0,1	2,8	≤0,1	≤0,1
G (D)	≤0,1	0,8	≤0,1	≤0,1

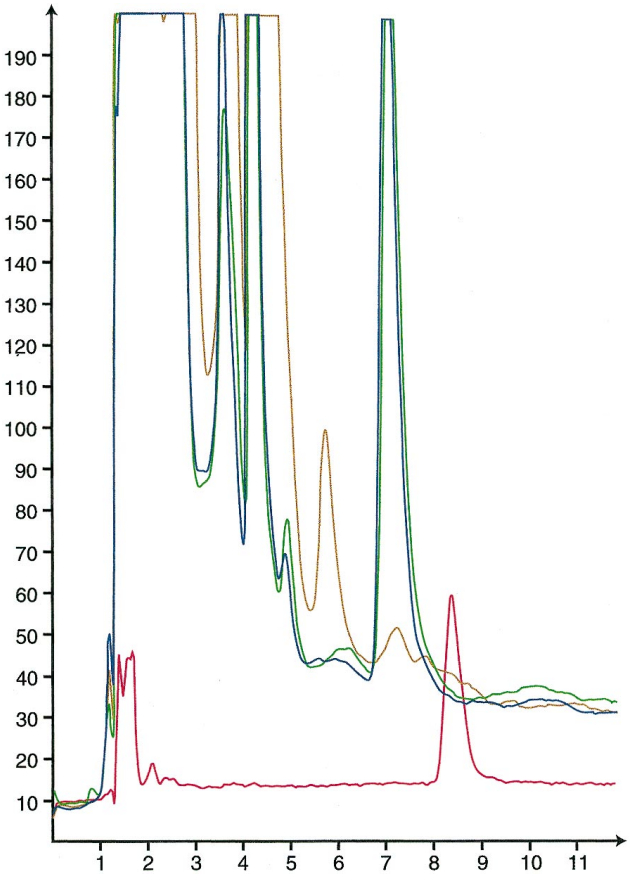


Fig. 2 Chromatogram showing elution profile of saliva samples collected before and after application of Visio-Seal fissure sealant (patient H). Baseline (brown); Immediately after fissure sealant placement (blue); 1 h after fissure sealant application (green); BPA standard, 66 ppb (red)

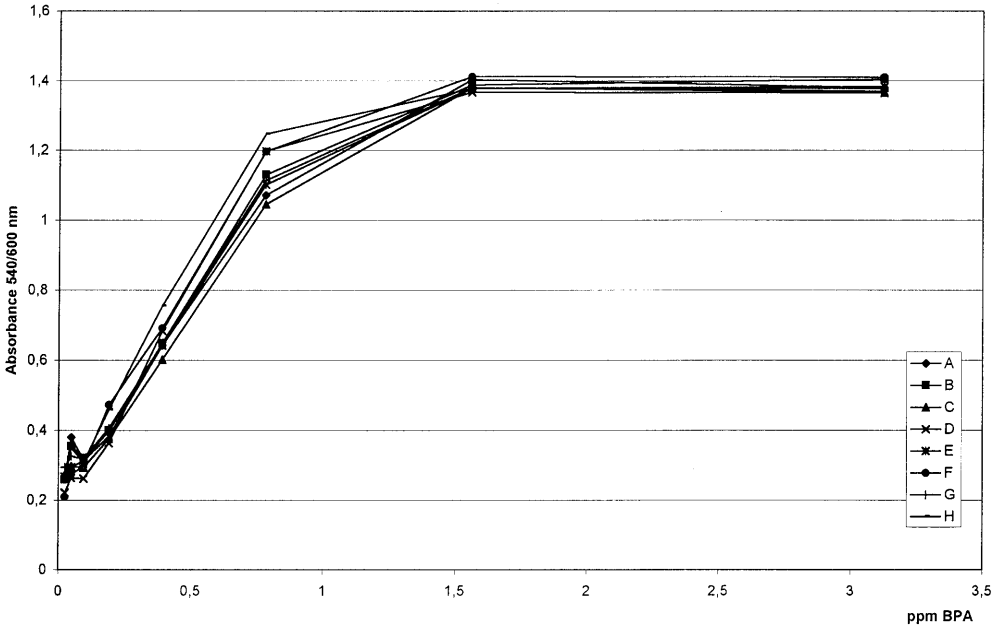


Fig. 3 Dose-response curve for estrogenic effect of BPA in the recombinant yeast cell human estrogen receptor transactivation assay. Letters refer to the individual patient series in which the data were obtained

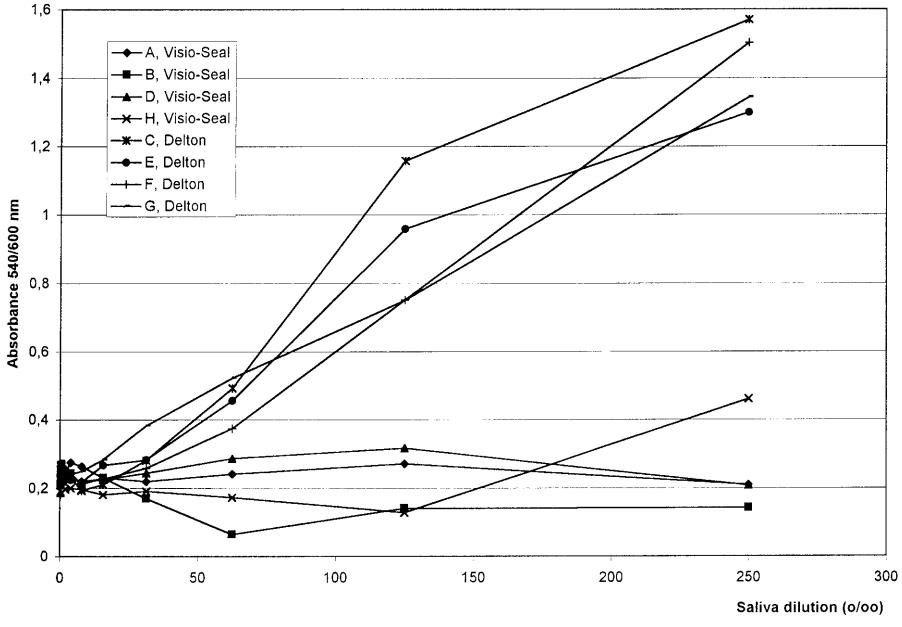


Fig. 4 Dose-response curve for estrogenic effect on dilutions of saliva samples collected immediately after application of fissure sealants

sealant show a peak with retention time concordant with the retention time of the BPA-standard (Fig. 1a-b). This did not occur in saliva samples received from patients immediately after placement of Visio-Seal fissure sealant (Fig. 2). Dose-response curves for the estrogenic effect of BPA in the yeast cell assay are given in Fig. 3, and showed a dose-related estrogenic effect within a dose-range relevant to the present study (maximum effective level 1.56 ppm BPA). Dose-response curves for the estrogenic effect of saliva samples collected immediately after placement of sealants are given in Fig. 4 and show a significant estrogenic effect in the 1:3 and 1:7 dilutions of saliva samples collected immediately after placement of Delton LC fissure sealant. No statistically significant effect was detected in the more diluted samples. The time-related estrogenic activity in the 1:3 diluted saliva samples collected from patients receiving Delton or Visio-Seal sealant respectively are shown in Figs. 5 and 6. The estrogenic activity in samples collected immediately after placement of Delton LC fissure sealant was significantly different from the control level (P<0,05).

Statistical analyses were performed by ANOVA and Bonferroni's multiple comparison test with acceptance of significance at P<0.05. Baseline data obtained from morning saliva samples (fasting) served as controls.

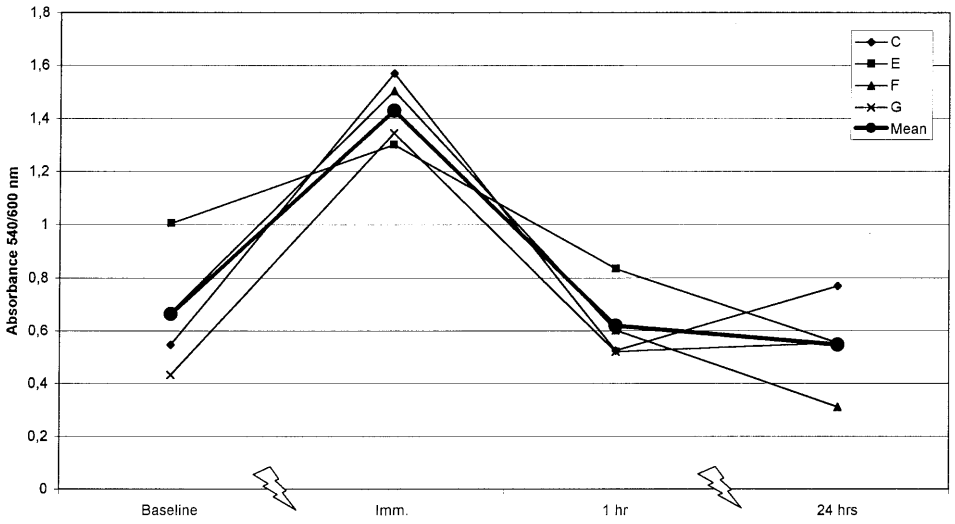


Fig. 5 Time-related estrogenic effect of saliva samples collected before and after application of Delton LC fissure sealant

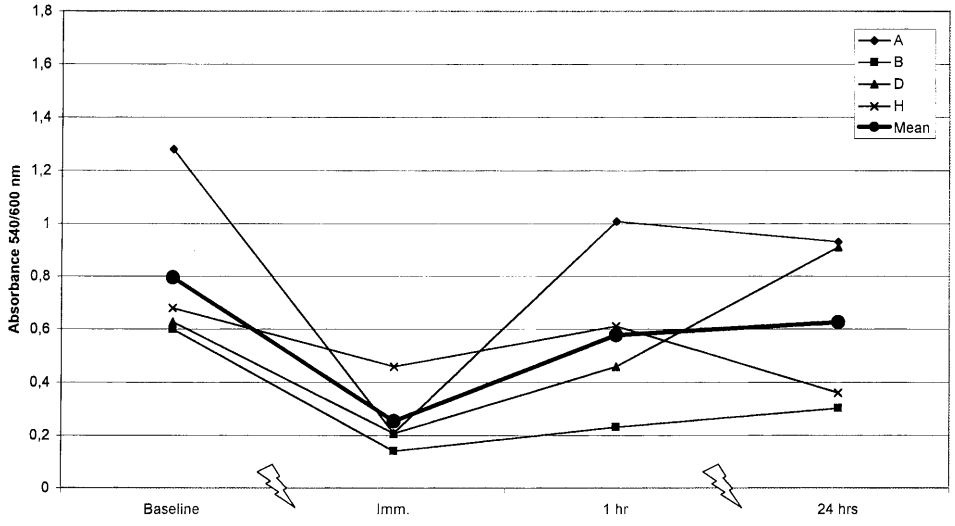


Fig. 6 Time-related estrogenic effect of saliva samples collected before and after application of Visio-Seal fissure sealant

Discussion

In the present study, the mean BPA-levels found in saliva immediately after and 1 h after application of Delton LC fissure sealant (1.43 ppm and ≤ 0.1 ppm respectively) were considerably lower than the BPA levels previously reported in saliva samples obtained 1 h after placement of Delton fissure sealant (mean 9.73 ppm)[9]. The slightly different amounts of fissure sealant applied in the two studies (mean 38±3 mg versus 50 mg [9]) may only be part of the explanation for the different BPA levels. Details about the clinical procedure whereby the fissure sealant was applied by Olea et al. are not clear [9]. It is therefore tempting to speculate that saliva samples may have been collected before removal of the unpolymerized surface layer which could add to the discrepancies between the BPA levels found in the previous and the present study. Furthermore, the data presented by Olea et al. were obtained from saliva samples collected 1 h after placement of fissure sealant, resulting in a total of 27–38 ml saliva per person. Our time-related data showed that immediately after but not 1 and 24 h after application of one fissure sealant, the BPA levels in saliva were above the detection limit of 0.1 ppm. BPA levels in saliva samples collected after application of Visio-Seal fissure sealant were all below the detection limit. These findings were confirmed by HPLC analyses from which peaks of BPA were detected in saliva samples collected immediately after application of Delton LC fissure sealant (Figs. 1 and 2) whereas no BPA peaks were found in the remaining samples (Fig. 2). Recent reports on in vitro elution of leachable components from dental sealants contradict the conclusion of Olea et al. regarding the leachability of BPA from dental sealants [5,8]. None of these studies detected BPA in eluates from any of the sealants tested – including Delton – whereas TEGDMA was identified as the principal eluted component [5,8]. It was emphasized that the methodology (e.g. elution time) may influence the interpretation of the peaks revealed by HPLC and attention was drawn to the fact that Olea et al.

did not identify TEGDMA release from Delton. Our study supports the recent in vitro reports that question the findings of high levels of BPA in saliva after placement of fissure sealant [9]. The discrepancies between BPA saliva levels found by Olea et al. and the levels detected in the present study may – apart from the different clinical procedures – also be explained by different interpretations of the identity of the components causing the peaks [5,8].

A recent study showed that Bis-DMA subjected to esterases or saliva resulted in a conversion of Bis-DMA into BPA [12]. We thus suggest that the BPA content found in the present study of saliva samples collected immediately after application of the Bis-DMA containing fissure sealant Delton LC may be caused by conversion of Bis-DMA into BPA by a degradation process mediated by esterases in saliva.

Our chemical analyses were confirmed by data obtained in the yeast cell assay. A significantly higher absorbance, indicative of estrogenic activity, was found in saliva samples collected immediately after placement of Delton LC fissure sealant (P <0.05). None of the remaining experimental groups differed significantly from the control group although a slightly decreased absorbance (non significant) was recorded in samples collected immediately after placement of Visio-Seal fissure sealant which may be caused by a slight cytotoxic effect of other substances e.g. EGDMA released from the product.

From the BPA dose-response curves(Fig. 3) it can be seen that the maximum level of estrogenic activity in the yeast cell assay was reached at a level of 1.6 ppm BPA. This is slightly above the mean BPA level found in saliva samples collected immediately after of Delton fissure sealant. The assay therefore tends to underestimate the estrogenic effect of BPA-concentrations above the level of 1.6 ppm (n=2) which are thus not fully reflected in the peak of estrogenic activity seen in Fig. 5.

In summary, the present study confirms that BPA can be found in saliva following placement of the fissure sealant Delton LC. However it is found in considerably lower amounts than previously reported and only immediately after placement of sealant. From 1 h after placement of the sealant, neither BPA nor estrogenic activity could be detected. On this basis, there seems to be no reason for concern about clinical implications of the short-term low level BPA exposure that may occur following application of Delton fissure sealant that is in accordance with a recent survey of the literature [14]. The study further showed that neither BPA nor estrogenic activity could be detected in saliva samples collected after placement of Visio-Seal fissure sealant.

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