

SUPPORTING INFORMATION

Bisphenol A in human saliva and urine before and after treatment with dental polymer-based restorative materials

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Appendix: Analysis of bisphenol A

Bisphenol A (BPA) and D16-BPA were obtained from Sigma-Aldrich Inc. (St. Louis, Missouri, USA). Acetonitrile (AcN) and ammonium acetate were from Merck (Darmstadt, Germany). Water was from a Milli-Q Integral 5 system (Millipore, Billerica, Massachusetts, USA). Serum (Fetal Bovine Serum) was from Gibco Thermo Fisher Scientific (Waltham, Massachusetts, USA). β -Glucuronidase (Escherichia coli K12) was obtained from Roche Diagnostics (Mannheim, Germany).

Preparation of standard samples and control samples (quality control)

Stock solutions were prepared by dissolving accurately weighed amounts of BPA in 50% AcN. Standard solutions were prepared by further dilution of the stock solutions in 50% AcN. Serum was used for the calibration standards for the saliva analysis and urine (obtained from healthy volunteers at our laboratory) for the urine analysis

Calibration standards were prepared by adding 25 μ l standard solution each to serum and urine.

Two control samples were used for quality control (QC). Samples were prepared by additions of small amounts of BPA into pooled urine samples.

Preparation of saliva samples

The samples were prepared in 96-well plates with 2-ml flat bottom glass vials (Biotech solutions, Vineland, New Jersey, USA). For the analysis of total BPA in the samples, aliquots of 100 μ l of saliva were added with 10 μ l glucuronidase and 10 μ l of 1M ammonium acetate buffer at pH 6.5. The samples were digested at 37°C for 90 min. Then, 25 μ l of 50% AcN containing D16-BPA as the internal standard and an additional 25 μ l of 50% AcN was added. Thereafter, the proteins were precipitated with 200 μ l acetonitrile followed by vigorous shaking for 30 min. For the calibration standards, aliquots of 100 μ l of serum were used and treated as above, but standards were added in 25 μ l of AcN. The samples were thereafter centrifuged at 2600 \times g for 10 min. The supernatant (0.2 ml) was transferred to a new 96-well plate with 0.5-ml conical glass vials (MicroLiter Analytical Supplies, Inc., Suwanee, Georgia, USA) for analysis and centrifuged again at 3000 \times g for 10 min before analysis.

Preparation of urine samples

The samples were prepared in 96-well plates with 1-ml glass inserts (1-ml SQW Micro-Inserts, La-Pha-Pack, Langerwehe, Germany). For the analysis of total BPA in the samples, aliquots of 200 μ l of urine were added with 10 μ l of glucuronidase and 100 μ l of 1M ammonium acetate buffer at pH 6.5. The samples were digested at 37°C for 30 min. Then, 25 μ l of 50% AcN containing D16-BPA as the internal standard and an additional 25 μ l of 50% AcN was added. For the calibration standards, aliquots of 200 μ l of urine were used and treated as above, but standards were added in 25 μ l of 50% AcN. The samples were thereafter centrifuged at 3000 \times g for 10 min.

Samples analyzed for free BPA in saliva were determined as above but digestion using glucuronidase was omitted. The concentration of conjugated BPA was estimated by the difference between total and free BPA.

Quantitative analysis

Quantitative analysis was conducted using triple quadrupole linear ion trap mass spectrometry (QTRAP 5500; AB Sciex, Foster City, California, USA) coupled to a liquid chromatography system (UFLCXR, Shimadzu Corporation, Kyoto, Japan; LC/MS/MS). Pure Nitrogen was used as the nebulizer, auxiliary gas, curtain gas, and collision gas. The temperature of the auxiliary gas was set at 630°C and the ion spray voltage was -4500 V. The MS analyses were carried out using selected reaction monitoring (SRM) in negative ion mode. BPA was analyzed using the transitions m/z 227-212 as the quantifier ion, m/z 227-133 as the qualifier ion, and m/z 241-142 was used for the internal standard.

In the LC system, a C18 column (4 μ m, 2.1-mm i.d. x 50-mm, Genesis Lightning; Grace, Hichrom, Reading, United Kingdom) was used prior to the injector to filter the mobile phases from contaminating BPA. Aliquots of 5 μ l of the samples were injected on a C18 column (same as above). The mobile phases were A: water and B: methanol, both containing 0.08% NH₃. The mobile phase was maintained at 5% B for 0.2 min after injection. A gradient was then applied in 7 min to 70% B and another 0.5-min increase of B to 95%. The column was then conditioned at 5% B for 1.5 min. A diverter valve was used and the column effluent was diverted to the MS between 4 and 7 min. The flow rate was 0.6 ml/min and the column was maintained at 45°C.

Concentrations were determined by peak area ratios between the analytes and the internal standard (IS). In addition, all values were corrected for the mean of the chemical blanks, which were run within each batch.

The limit of detection (LOD) was calculated as three times the standard deviation of the ratio between the peak area in the chemical blank samples at the analyte retention time and the peak area of IS, divided by the slope of the calibration line. The LOD was determined to be 0.1 ng/ml for both saliva/serum and urine analysis.

In all analytical batches there were two different in-house prepared QC samples and chemical blanks analyzed. The samples were prepared and analyzed in duplicates and the mean of the two concentrations was used.

Between-run precision was estimated from the above QC samples analyzed in 76 batches of urine samples. The CV of the two QC samples was at 2.6 ng/ml 14% and at LOD at 0.1 ng/ml 42%.

The within-run precision was determined in spiked serum samples (n=10) at three levels. The CVs were at 13 ng/ml 2.8%, at 7 ng/ml 3.4% and at 2 ng/ml 4%. The laboratory in Lund performing the analyses is a European reference laboratory for BPA in urine (www.eu-hbm.info/democophes) and a reference laboratory for BPA in urine in the Erlangen Round Robin inter-laboratory control program.

Qualitative analysis

Because, in the HPLC-MSMS run of the saliva samples, there was a small shift in the retention time between the BPA peak in the standard samples and the BPA peak in the saliva samples, a more thorough investigation was made to be able to confirm that the peaks in the saliva samples originate from BPA. The samples were also analyzed on a quadrapole time of flight mass spectrometer (QTOF; Triple TOF 5600; AB Sciex, Foster City, CA, USA) coupled to a liquid chromatography system (UFLCXR, Shimadzu Corporation, Kyoto, Japan).

The columns and mobile phases including the settings were the same as for quantitative analysis. The mobile phase was kept at 5% B for 0.5 min after injection. A gradient was then applied in 3.5 min to 95% B and was maintained for 1 min. The column was then conditioned at 5% B for 1.5 min. The column effluent was diverted to the MS between 3.0 and 4.7 min.

The temperature of the auxiliary gas on the MS was 600°C and the ion spray voltage was -4500 V. The MS analysis was carried out using product ion scan in negative ion mode. For accurate mass determination, BPA was analyzed using the precursor ions m/z 227.1 for BPA, and m/z 241.1 was used for the BPA-d16 internal standard.

The QTOF spectra of the chromatographic BPA peak from the standard were compared with the QTOF spectra of the chromatographic BPA peak from the saliva samples. The spectra found in the standard sample were comparable to the peak found in saliva, thus suggesting that the peak in the saliva samples is an isomer of BPA and this causes a small shift in the retention time.

Fig. S1. Total ion chromatogram for the BPA peak from the standard sample of 50 ng/ml (retention times: BPA, 3.36 min (black); BPA-d16, 3.34 min (gray))

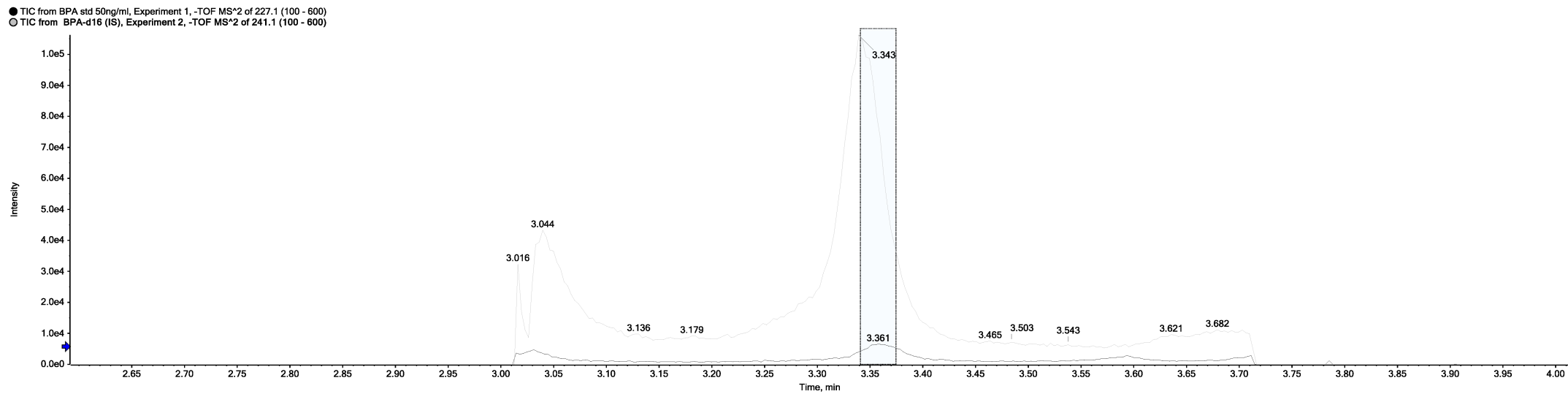


Fig. S2. Quadrupole time of flight mass spectra for the BPA peak from the standard sample of 50 ng/ml (retention time: 3.36 min).

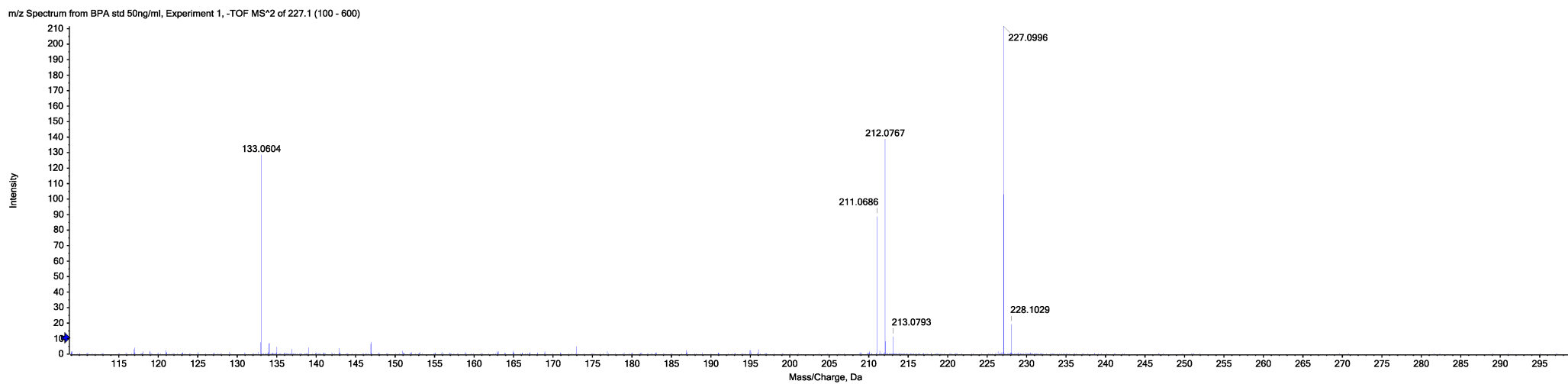


Fig. S3. Total ion chromatogram for the BPA peak from a saliva sample with a concentration of 800 ng BPA/ml (retention time: BPA, 3.40 min (black); BPA-d16, 3.34 min (gray)).

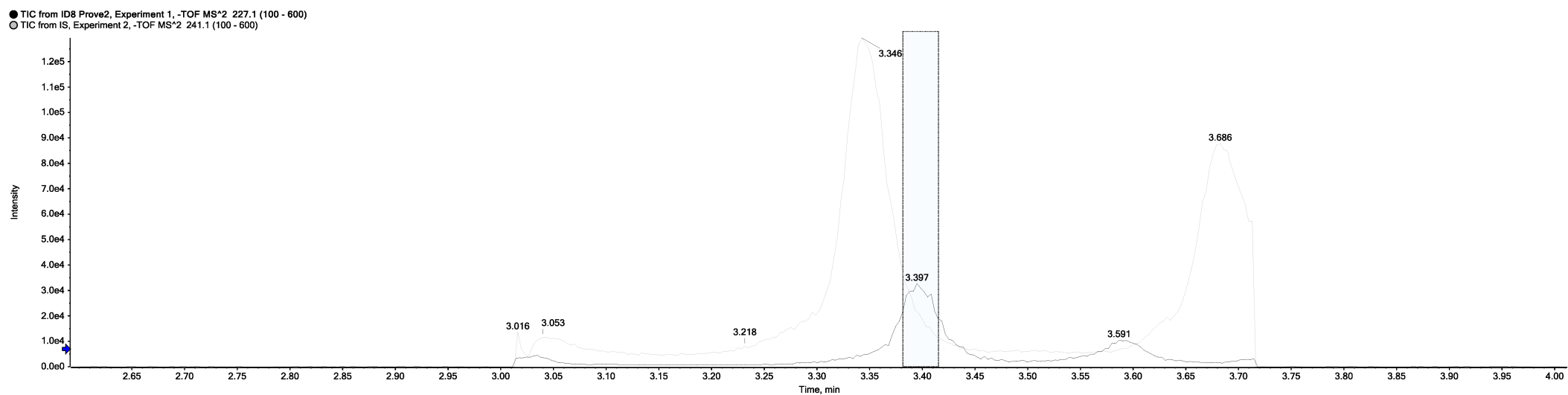


Fig. S4. Quadropol time of flight mass spectra for the BPA peak from the saliva sample shown in Fig. 3 (retention time: 3.40 min).

