LETTER

Multi-class Analysis of Exposure Chemicals in Deciduous Teeth by Liquid Chromatography-Mass Spectrometry: Preliminary Studies on Sample Preparation Methods

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Abstract : Since accumulation of chemicals in deciduous teeth can occur from the second trimester of fetal development to shedding, a deciduous tooth has been considered as an attractive biomatrix for estimating individual chemical exposures recently. Therefore, detection of organic chemicals from teeth has received an increasing attention in exposomics research. Most previous studies on organic chemical analysis of teeth not only focused on a few targeted chemicals but also ignored potential contaminants from an enamel surface or a dental pulp. Recently, our group started developing a multi-class organic analysis method for deciduous teeth and tried to find a proper incubation condition of tooth materials. Our results showed that incubation with methanolic HCl provided the best performance among tested.

Keywords : exposomics, deciduous teeth, sample preparation, dentin, enamel

Introduction

Exposomics tries to assess all the environmental exposures of an individual and to reveal how the measured exposures affect to human health.[1] As a promising biomatrix for exposomics research, deciduous teeth have received a great attention due to their unique features.[2] Since deciduous tooth development starts from the second trimester of fetal development and continues until shedding, chemicals accumulated in a single deciduous tooth are from both prenatal and postnatal period. In addition, chemicals accumulated in a prenatal period separated from those in a postnatal period by a neonatal line formed in deciduous teeth due to its specific dentin growth direction. Therefore, there are increasing needs for analyzing chemicals present in a tooth matrix with high

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spatial resolution and with good sensitivity.

Although a tooth matrix is one of the highly mineralized tissues, elemental analyses with teeth can be achieved rather easily by employing either solublization of a tooth material with a concentrated acid or ablation sampling with an intense pulsed laser because chemical decompositions are not a great concern in elemental analysis.[3,4] However, analysis of organic compounds present in a tooth faces much more challenging tasks than elemental analysis. First, contamination from pulp tissues and blood should be avoided because abundances of organic chemicals in pulps or blood are much higher than those in mineralized dental tissues such as enamel, dentin, and cementum.[5] Second, conditions for incubating tooth materials and extracting organic chemicals from a tooth matrix should be harsh enough to release organic chemicals from a highly mineralized hydroxyapatite network, but not too harsh for organic chemicals to be decomposed or hydrolyzed.

With a tooth matrix including both deciduous and permanent tooth samples, most of previous studies conducted organic chemical analyses for a few target analytes. Target analytes studied include polychlorinated biphenyls (PCBs),[6-9] psychotropic drugs,[10-12] environmetal tobacco smokes,[13-15] antibiotics,[16-17] analgesic drugs,[18,19] and phthalates.[18,19] Therefore, each study has a different sample preparation protocol from each other. For analyzing PCBs, teeth were incubated in concentrated sulfuric acid

and hexane with an aid of ultrasonication.[1-3] For lidocaine, nicotine, and cotinine, teeth were subjected to incubation with 1 to 2 M sodium hydroxide (NaOH).[10,13,15] For opiates and cocaine, 0.1 M HCl was the incubation solution for teeth.[12] However, these incubation methods have never been tested against multi-class organic chemicals. Recently, an analysis platform for profiling multi-class organic compounds from teeth was suggested by Arora et al.[20] Their method included tissue sampling by laser capture microdissection, sequential extraction with glacial acetic acid in acetonitrile (ACN) and ammonium hydroxide, preconcentration with solid phase extraction, and liquid chromatography/mass spectrometry (LC/MS) analysis. Although various classes of exposure chemicals were detected in that study, there was no systematic evaluation on their sample preparation steps and possible contamination sources.[20]

In this work, we performed preliminary studies on sample preparation steps for multi-class organic chemical analysis of deciduous teeth. Decontamination and cleaning procedures were investigated by matrix-assisted laser desorption/ionization (MALDI) MS. In addition, we tested several incubation conditions by performing an LC/MS-based, multi-class organic chemical screening analysis which can detect eighty two exposure chemicals from seven different classes such as phenols, phthalates, and so on.[21] Our results showed that incubation with methanolic HCl provided the best performance among tested.

Experimental

Materials

Methanol, LC/MS-grade water, and LC/MS-grade ACN were purchased from Fisher scientific (Fairlawn, NJ, USA). Trifluoroacetic acid (TFA), *tert*-Butyl methyl ether (MTBE), ethyl acetate, potassium carbonate, dichloromethane (DCM) and 2,5-dihydroxybenzoic acid (DHB) were obtained from Sigma-Aldrich (St. Louis, MO, USA). β-glucuronidase was purchased from Roche (Basel, Switzerland).

Deciduous teeth with no dental caries were obtained in a non-invasive way at local dental clinic. Donated teeth were stored in distilled water before sample preparation. Sample preparation workflow is outlined in Figure 1. For decontamination, pulp tissues and other surface residues were first removed by using a sickle scaler. Then, teeth were subjected to two times 15 min-sonication cleanings in distilled water. After each sonication, a washed solution was collected separately for MALDI MS analysis. After sonication, washed teeth were swirled in DCM for 5 min to ensure complete removal of external organic chemicals on a tooth surface. Decontaminated, dried teeth were then pulverized by using a ball-mill grinder (Pulverisette 23, Fritsch GmbH, Idar-Oberstein, Germany) and a prepared pooled teeth powder was stored in a glass vial at room temperature until incubation.
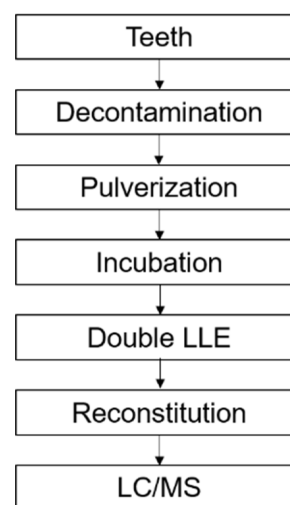
Sample collection and preparation

Figure 1. Experimental workflow for analysis of organic chemicals present in deciduous teeth.

Ten miligram aliquots of a pooled teeth powder were incubated in five different conditions: In (1) 0.1 M HCl at 37°C for 18 h, (2) 0.1 M NaOH at 37°C for 18 h, (3) 0.1 M HCl at 50°C for 1 h with sonication, (4) 0.1 M NaOH at 50°C for 1 h with sonication, and (5) 0.1 M 70% methanolic HCl at 50°C for 1 h with sonication. After incubation, solution pH was adjusted to about pH 7 for enzymatic hydrolysis of glucuronides by a β-glucuronidase. All incubation solutions except 0.1 M 70% methanolic HCl were directly subjected to the pH adjustment. In case of incubation with 0.1 M 70% methanolic HCl, methanol was first evaporated under N_2 and then the pH adjustment was achieved with phosphate buffer (pH 7.2).

Extraction of organic chemicals from incubation solutions was performed by a double liquid-liquid extration (dLLE) developed by Lee et al with minor modifications.[21] Briefly, solutions were alkalinized with 5 wt% K_2CO_3 and extracted with MTBE for 10 min with shaking. A solution was then centrifuged at 2500 rpm for 5 min and an organic solvent layer was collected. Remaining aqueous layer was acidified with 6 M HCl and extracted with ethyl acetate for 10 min. A solution was then centrifuged at 2500 rpm for 5 min and an organic solvent layer was collected and merged with the previously collected organic extract. A merged organic extract was dried and reconstituted with mobile phase solvent (A/B 98:2) used for LC/MS analysis.

MALDI MS analysis of washing solutions

For MALDI MS analysis of washing solutions, a DHB matrix solution (20 mg/mL) was prepared with 0.1% (v/v) TFA plus 1 mM NaCl in water/ACN (7:3, v/v). A washing solution (1.0 μL) was first spotted onto a MALDI target

plate (ASTA Inc., Suwon, Korea) followed by a 1.0 μL DHB matrix. The prepared sample spots were dried and MALDI MS analysis was performed with an ABI 4800 Plus MALDI-TOF/TOF analyzer (Applied Biosystems, Foster City, CA). Mass spectra were collected in the positive ion reflectron mode with a 20 kV acceleration voltage.

LC/MS analysis

LC/MS analyses were performed by a Shimadzu ultra fast liquid chromatography system (Nexera XR, Tokyo, Japan) interfaced with a Thermo Exactive orbitrap mass spectrometer with an electrospray ionization source (Thermo Scientific, Bremen, Germany). LC separation was achieved with a Phenomenex Kinetex C18 column (2.0 mm id × 100 mm, 2.6 μm particle size, Torrence, USA). The mobile phases were (A) 5 mM acetic acid in water and (B) 5 mM acetic acid in ACN. The solvent program (gradient) consisted of holding solvent (A/B 98:2) for 0.5 min, the linearly converting to solvent (A/B 5:95) for 8 min, holding solvent (A/B 5:95) for 0.5 min, followed by re-equilibration. The flow rate was 500 μL/min and the injection volume was 20 μL.

Results and Discussion

Decontamination

After collection, a tooth sample should undergo a proper cleaning process in order to minimize external organic contamination including blood and any surface residues. In general, a hydrogen peroxide solution is the most frequently used cleaning solution in a dental clinic to remove blood from collected teeth. However, we did not consider a hydrogen peroxide solution as a decontamination or washing solution because it could obviously induce severe oxidation of organic chemicals present in a tooth. Therefore, we tested other solutions used in previous studies[6-19] such as distilled water, a saline solution, a sodium hypochlorite (NaOCl) solution, and DCM. Among tested, saline and sodium hypochlorite solutions are found to be inappropriate due to oxidation or high salt concentration (data not shown). Therefore, we decided to employ a sequential cleaning procdure with distilled water and DCM.[19]

After each cleaning step, a washing solution was collected and its chemical fingerprint was obtained by MALDI MS. As shown in Figure 2(a), a group of peaks originated from a surfactant were detected with 44 Da spacings from the first distilled water washing solution. The second washing solution also showed surfactant peaks, but with substantially reduced intensities. Figure 2(b) showed a chemical fingerprint of a DCM washing solution and there was no noticable contaminant peak in this solution. It should be noted that absence of a contaminant peak in MALDI mass spectra is not a sufficient evidence of complete decontamination. Further evaluation of
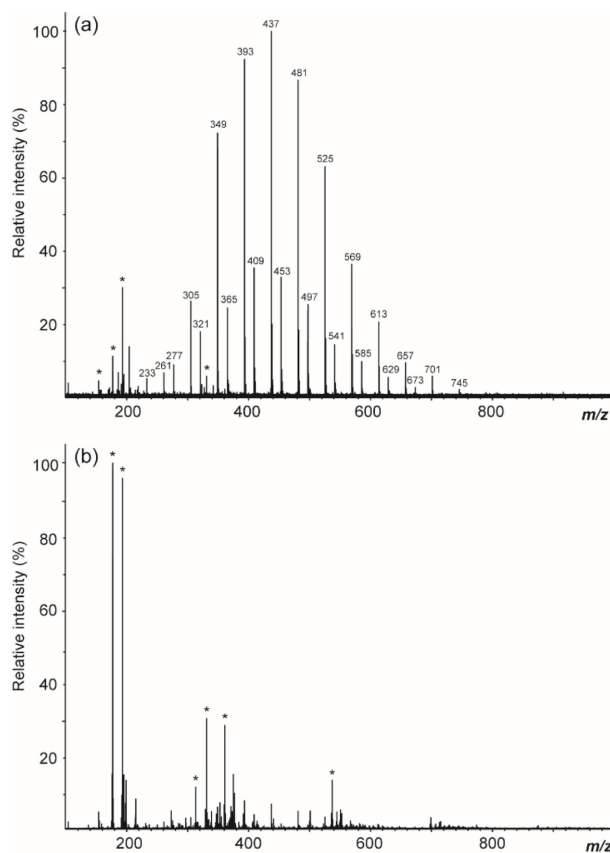

Figure 2. MALDI mass spectra of (a) the first washing solution and (b) the DCM washing solution. Peaks with asterisks (*) are DHB matrix-originated ion signals.

cleaning procedure will be conducted with a LC/MS analysis in near future.

Incubation

Incubation, a process of releasing analytes of interests from a biomatrix, is a critical sample preparation step for solid biomatrices. Since organic chemicals in a tooth are thought to be trapped in a highly mineralized, collagen-hydroxyapatite composite, incubation of a tooth matrix is the most challenging part among sample preparation steps. In the beginning of investigation, we tried to achieve complete solubilization of a tooth matrix by employing a concentrated acid. Although complete solubilization of a tooth matrix was achieved with overnight incubation in 0.7 M nitric acid, we could not detect any relevant organic chemicals from this treatment. Therefore, we decided to test rather mild acidic or basic incubation conditions as described in Experimental section. In order to evaluate performances of incubation conditions, we performed an LC/MS-based screening analysis.[21] First, we set up an LC/MS method which can detect and identify major 82 exposure chemicals by matching accurate masses and retention times. Eighty two
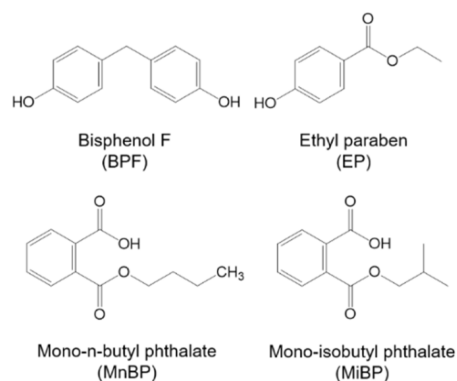
Figure 3. Chemical structures of organic chemical compounds detected from a pooled deciduous teeth powder.

Figure 4. (a) Extracted ion chromatograms (EICs) of major compounds detected from a pooled teeth powder which was incubated in 70% (v/v) methanolic HCl. (b) EICs of corresponding compounds present in a positive control.

target analytes are composed of seven classes of organic chemicals, 25 volatile organic compounds (VOCs), 34 phenols, 14 phthalates, 2 pyrethroids, 2 environmental tobacco smoke compounds, 5 polyaromatic hydrocarbons (PAHs). Second, all samples incubated in different conditions were subjected to the same dLLE procedure and the LC/MS screening analysis. In addition, a positive control which contains 82 standard chemicals and a negative control were also processed in an exactly same way.

Figure 3 shows four chemical structures of organic chemical compounds found in a pooled deciduous powder, and Figure 4 shows extracted ion chromatograms (EICs) of these four compounds in a teeth sample incubated with 70% methanolic HCl and in a positive control. Besides listed compounds, benzophenone-3, monoethyl phthalate, *para*-nitrophenol, propyl paraben, and 4-hydroxybenzoic acid were also detected from some samples, but these compounds were excluded because they were found to be susceptible to contamination from current sample preparation steps.

Bisphenol F (BPF) has been detected in everyday products such as toothpastes and also used in dental materials such as dental sealants.[22] Ethyl paraben (EP), an ethyl ester of *para*-hydroxybenzoic acid, has been widely used as a preservative in foods, pharmaceuticals, and cosmetics.[23] Mono-*n*-butyl phthalate (MnBP) and mono-isobutyl phthalate (MiBP) have been commonly used as plasticizers and easily found in various consumer products.[24] Therefore, these four compounds may accumulate in teeth through oral intake or inhalation.

Among tested incubation conditions, acidic incubations generally gave the better results than basic ones in terms of detectibility and sensitivity. From the samples incubated in NaOH, only BPF was clearly detected. In contrast, all compounds were detected from the samples incubated in HCl, but only the incubations under 0.1 M HCl at 37°C for 18 h and under 0.1 M 70% methanolic HCl at 50°C for 1 h with sonication gave the signals of EP, MnBP, and MiBP intense enough to be clearly distinguished from
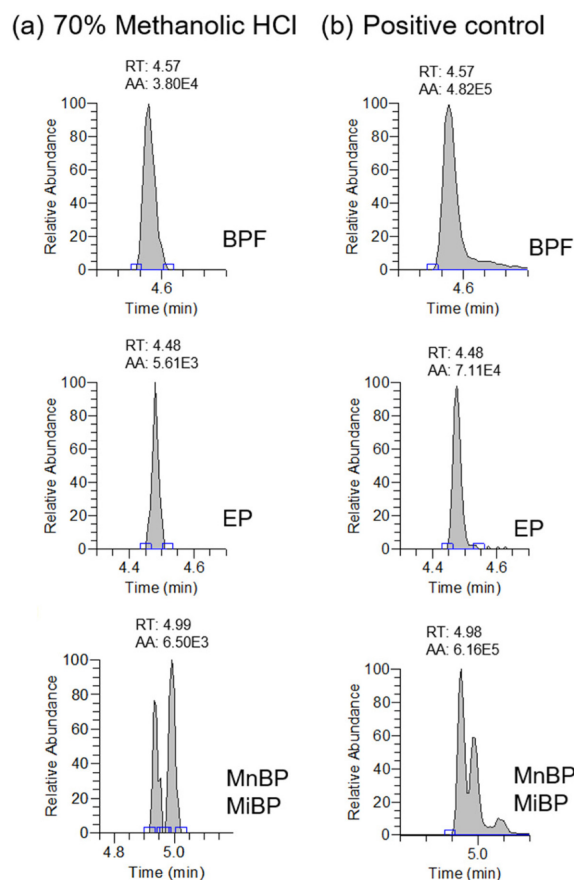
background signals. Therefore, under current experimental conditions, incubation of a tooth matrix with 70% methanolic HCl provided the best results in terms of sensitivity and speed of analysis.

Conclusions

In this study, preliminary optimization of sample preparation steps was performed for multi-class analysis of organic exposure chemicals from deciduous teeth. Under the current experimental set up, a sonication-aided decontamination with a combination of distilled water and DCM seemed to be appropriate for deciduous teeth analysis. In addition, methanolic HCl incubation followed by dLLE gave the best results so far. However, the suggested decontamination and incubation steps derived in this study are preparatory and based on small number of experimental conditions. Therefore, more systematic and comprehensive evaluation is required for a more reliable tooth organic analysis platform.

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Acknowledgments

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