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Simultaneous determination of BTEX and their metabolites using solid-phase microextraction followed by HPLC or GC/MS: An application in teeth as environmental biomarkers

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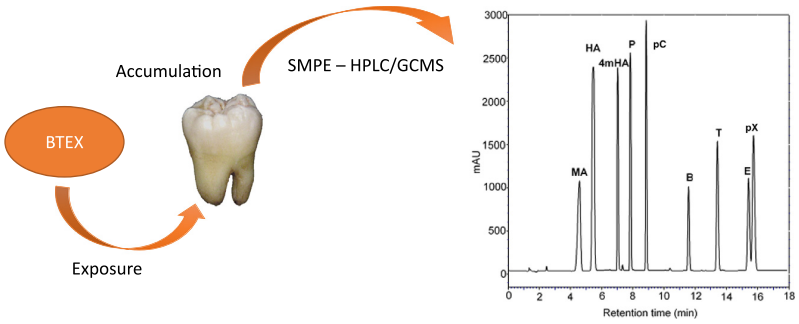
HIGHLIGHTS

- A new method for the analysis of BTEX and its metabolites in dental tissues is proposed.
- It consists in a one-step extraction and SPME-HPLC or GC–MS determination.
- The new methodology has adequate precision, exactitude and detection limits.

GRAPHICAL ABSTRACT



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ABSTRACT

Applications of benzene, toluene, ethylbenzene, and *o*-, *m*-, and *p*-xylenes (BTEX) release them into the environment exposing living organism. These endocrine disruptors are toxic, highly volatile and easily absorbed by the lungs and can cause adverse consequences for the human health as neurological diseases and cancer.

A method for the analysis of BTEX and its metabolites (phenols and aromatic acids) in teeth is presented. The method consists in a one-step simple extraction procedure from spiked tooth using NaOH solution followed by SPME-HPLC or HS-SPME-GC/MS determination. Optimization of both, spiking procedure and extraction step of these analytes from tooth, was carried out. Two fibers CAR/PDMS for BTEX and PA for BTEX metabolites were used for the SPME and variables were optimized for analytes at 30 °C using spiked solutions. The optimized adsorption times were 30, 75 and 30 min and desorption times were 10, 40 and 30 min for BTEX, phenols and aromatic acids, respectively. Linearity for SPME-HPLC method was established using spiked solutions with both, BTEX and metabolites, at 2.5, 5.0, 10.0, 25.0 μg/mL. The obtained results indicated a good linearity (r^2 above 0.994) for all analytes. Triplicate analyses were performed with RSD lower than 15%. LODs were in the range 0.2–33.3 ng/mL for SPME-HPLC and 0.06–0.09 pg/mL for HS-SPME-GC/MS methods in spiking solutions. Once the method was optimized, bovine teeth were used as biological matrix model for the tuning of spiking and extraction steps. Optimal adsorption and desorption times were 4 h for both procedures. Micrograms per tooth gram of BTEX and phenols were quantified in ten human teeth and aromatic acids were not identified.

The developed method for BTEX and metabolites analyses using SPME-HPLC or HS-SPME-GC/MS shows good precision, linearity and sensitivity. The method was successfully applied in human teeth as environmental biomarker of BTEX and metabolites.

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1. Introduction

Benzene, toluene, ethylbenzene, and *o*-, *m*-, and *p*-xylenes (BTEX) are widely used not only as industrial raw materials but also as solvents, primary components of motor vehicle gasoline and starting products in a variety of chemical syntheses. These applications have released BTEX to the environment exposing living organisms to them (Esteve-Turrillas et al., 2007; Słomińska et al., 2014). As far as human exposition is concerned, industry workers and, in particular, gas station employees are occupational exposed to this family of compounds (Moolla et al., 2015). These highly toxic volatile organic compounds are easily absorbed *via* the lungs as well as the skin which may also be a significant absorption route (Reese and Kimbrough, 1993). Human exposition to these substances can cause adverse consequences for the human health by such as neurological diseases or cancer (Hinwood et al., 2007; Yimrungruang et al., 2008). A recent review has shown that BTEX may have endocrine disrupting properties at exposure levels even below the reference concentration (Bolden et al., 2015).

Phenols are metabolites of benzene and its alkyl derivatives (Bieniek, 1994). Primarily, alkylbenzenes are oxidized at the alkyl side chain, which in turn, give rise to aromatic carboxylic acids. Hippuric acid (HA) is the chief metabolite of toluene, mandelic acid (MA) and phenylglyoxylic acid (PGA) are major metabolites of ethylbenzene, and 2-, 3-, 4-methylhippuric acid (mHA) are metabolites of xylene isomers (Angerer and Hörsch, 1992). Thus, the assessment of BTEX metabolite concentrations on biological materials permits the degree of exposure.

HPLC, GC and capillary zone electrophoresis (CZE) are used to determine BTEX and their metabolites in biological fluids, such as urine and blood, when monitoring workers exposition (Aranda-Rodriguez et al., 2015; Fustinoni et al., 2010; Hrivňák and KráĽovičová, 2009; Wang et al., 2003). HPLC methods facilitate the simultaneous determination of MA, PGA, mHA metabolites (Moro et al., 2010; Yang and Liu, 2010), and the analysis time is usually between 20 and 50 min. The retention time for gas chromatography methods is shorter but the main disadvantage of GC methods is the need of an extraction step with an additional derivatization procedure that makes the determination more laborious and time consuming. CZE is found to be superior to HPLC and GC; analysis time is much shorter (10 min) (Wang et al., 2003). However, this equipment is not usually present at analytical laboratories as HPLC or GC are.

When determining traces of these compounds in liquid and solid samples a pre-concentration step previous to the chromatographic analysis is necessary. Solid-phase microextraction (SPME) and headspace-SPME (HS-SPME) are rapid, selective, easily automated and solvent-free techniques extensively applied for the analysis of compounds in complex matrixes like food and environmental (soils, sediments and wastewaters) (Ouyang and Pawliszyn, 2006; Merkle et al., 2015; Souza-Silva et al., 2015). In the SPME optimization studies for BTEX, phenols and aromatic acids determination, the best results are obtained in liquid samples (Ezquerro et al., 2004) at pH 2 and saturated with NaCl (van Doorn et al., 1998; Baciocchi et al., 2001). The studies of the temperature effects in the SPME for these analytes demonstrates that the optimum extraction efficiency is achieved in the range 20–30 °C (Ezquerro et al., 2004; Lee et al., 2007).The determination of BTEX (Ezquerro et al., 2004; Lee et al., 2007), phenols (van Doorn et al., 1998; Baciocchi et al., 2001; Buchholz and Pawliszyn, 1994; Barták and Čáp, 1997), and aromatic acids (Fan and Deng, 2002; Spietelun et al., 2011) by SPME followed by GC, HPLC or CZE techniques allows satisfactory results with high separation efficiency, simplicity, low injection volume, and short analytical time. See Table 1 for a comparison for these methods.

Body fluids are adequate biomarkers to assess exposure to BTEX during short periods of time, meanwhile bone and teeth are known to be historic markers. Since the use of bone as biomarker of exposure is difficult, collection of dentin, especially from 3rd molars (commonly extracted) is more accessible. Methods for the analysis of different compounds accumulated in teeth are found in the literature such as heavy metals, mainly lead, cadmium, zinc and manganese, that are sequestered by mineral phase of teeth during their formation (Gerlach et al., 2000; Nowak and Chmielnicka, 2000; Tvinnereim, 2000; Ericson et al., 2001; Kang et al., 2004; Arora et al., 2006; Costa de Almeida et al., 2007), dioxin in mammals teeth as biomarkers (Murtomaa et al., 2007), fluoride determination of human teeth used as a biomarker of fluoride exposure (Mehta, 2013) and nicotine and cotinine that were studied in teeth for monitoring cumulative exposure to environmental tobacco smoke (Pascual et al., 2003; Marchei et al., 2008). Up to now, the use of teeth as biomarkers of cumulative human exposure to BTEX has not been considered.

The aim of this study is to develop a method for consecutive determination of BTEX and metabolites in human teeth to obtain a promising and future tool for categorizing cumulative exposure to BTEX and its relation with dental health. A SPME-HPLC method for simultaneous analysis of BTEX, phenols and aromatic acids is investigated in spiked solution at pH 2 and saturated with NaCl using two different SPME fibers coated with carboxen-polydimethylsiloxane (CAR/PDMS) and polyacrylate (PA) to establish a methodology for determining the level of environmental exposure to BTEX, using dental samples as biomarkers. A HS-SPME-GC/MS method for BTEX analysis is also investigated. The optimization of the spiking and extraction step of BTEX and metabolites is investigated from spiked teeth, and the optimization of SPME variables, such as the adsorption and desorption times, is also studied for all analytes at 30 °C in spiked solutions. Finally, the features of both methods, SPME-HPLC and HS-SPME-GC/MS, are established and used to determine BTEX and metabolites in human teeth.

2. Experimental

2.1. Chemicals and samples preparation

Acetonitrile (Chromasolv grade), methanol (Chromasolv grade) and chloramine T were supplied by Sigma-Aldrich (Madrid, Spain). Anhydrous potassium dihydrogenphosphate (KH_2PO_4, 99.5%) were obtained from Merck (Hohenbrunn, Germany). Trisodium phosphate ($Na_3PO_4 \cdot 12H_2O$, 99.5%), acetic acid glacial and 85% orthophosphoric acid were obtained from Panreac (Madrid, Spain). Deionised water was purified through a Millipore purification system from Millipore (Milford, MA, USA). The following chemicals were used to prepare stock solutions in methanol: benzene (99.8%) from SDS (Peypin, France), toluene (≥99%) from Merck, ethylbenzene (≥99%), *p*-xylene (99%), chlorobenzene (99%), phenol (99.5%), *p*-cresol (99%), (*R*)-(−)-mandelic acid (98%), hippuric acid (≥97%), and 4-methylhippuric acid (98%) from Sigma-Aldrich (Madrid, Spain).

In order to reduce losses by evaporation of BTEX and phenols, the standard solutions were prepared, just before each study and chromatographic analysis, in sealed 20 mL- or 250 μL-vials without free headspace since BTEX and phenols migrate easily from solution to the headspace. The stock solution was prepared by dissolving appropriate amounts of each standard in methanol/water (1:1). Spiked dilutions were prepared in 20 mL-vials containing 10 mL of 1 M NaOH solution. These spiked solutions were used to optimize the SPME parameters and for the calibration of SPME-HPLC and SPME-GC/MS methods.

Bovine teeth were used as biological matrix model for the optimization of spiking and extraction steps. These teeth were previously stored in 1% chloramine T aqueous solution to avoid tooth degradation. Human teeth were kindly provided by dental service of CAP Drassanes, ICS (Barcelona) between October and November 2008. Individual human tooth was washed with water by the dentist post-extraction, and placed in 20-mL glass vial containing 1 M NaOH solution for the extraction process. Age, profession and smoking characteristic were determined for each donor.

Table 1
Comparison for different methods of SPME for benzenic compounds.

References	Analytes	Sample	Extraction	Fiber type	Chromatography
(Esteve-Turrillas et al., 2007)	Benzene Toluene Ethylbenzene *o*,*m*,*p*-Xylene	Soils	HS-SPME	CAR-PDMS	GC-FID CP-Select 624 CB; 30 m × 0.25 mm i.d. with 1.4 μm fused silica stationary phase
(Słomińska et al., 2014)	Phenol 2,3-Dichlorophenol 2-Nitrophenol 2,4,5-Trichlorophenol Pentachlorophenol	Water	pH 2 or 12 SPME extraction CCl_2H_2	100 μm PDMS 85 μm PA 65 μm CW–DVB	GC-FID HP-5 column 25 m × 0.33 mm i.d with a 0.17 mm film
(Moolla et al., 2015)	Phenol 3-Chlorophenol	Soils	pH 1, 60 min Room temp	CW–DVB	GC–FID BP-5 column 25 m ×0.33 mm i.d with 5% phenyl polysiloxane
(Reese and Kimbrough, 1993)	Benzene Toluene Ethylbenzene *o*,*m*,*p*-Xylene	Water	HS-SPME 15 min 25 °C 267 g/LNaCL	75 μm CAR-PDMS	Cryotrap-GC–MS DB-5 MS 30 m 30 m × 0.25 mm I.D., 0.25 μm stationary phase thickness fused-silica
(Hinwood et al., 2007)	Phenol 2-Chlorophenol 2-Nitrophenol 2,4-Dimethylphenol 2,4-Dichlorophenol 4-Chloro-3-methylphenol 2,4,6-Trichlorophenol 2,4-Dinitrophenol 4-Nitrophenol 2-Methyl-4,6-Dinitrophenol Pentachloropheno	Extracts	SPME In syringe	CAR-PDMS	GC-FID GC–MS PTE-5 30 mx0.25 mm i.d. 0.25-μm film thickness
(Yimrungruang et al., 2008)	Phenol 2-Chlorophenol 2-Nitrophenol 2,4-Dimethylphenol 2,4-Dichlorophenol 4-Chloro-3-Methylphenol 2,4,6-Trichlorophenol 2,4-Dinitrophenol 4-Nitrophenol 2-Methyl-4,6-Dinitrophenol Pentachloropheno	Synthetic	HS-SPME 60 min 30% NaCL 0.1 M HCl	100 μm PDMS 85 μm PA	GC-FID DB-5 column 30 m × 0.53 mm i.d., 1.5 μm
(Bolden et al., 2015)	3,4,5-Trimethoxybenzoic acid 4-Hydroxyphenylacetic acid Salicylic acid Ferulic acid p-Coumaric acid Vanillic acid 4-Hydroxybenzoic acid	Sediment	SPME immersion pH 2 20 °C	85 μm PA	CZE UV detector Fused-silica Capillary (57 cm × 75 mm I.D.)

Bovine clean teeth were immersed in 20-mL glass vials sealed with aluminum caps with 3.0 mm thick PTFE septa (Sugelabor, Madrid, Spain), containing standard solution (25 μg/mL) without headspace. The teeth were shaken using an ultrasonic bath (Branson 1210, Missouri, USA) and the kinetic of absorption of BTEX and metabolites was studied by duplicate at 2, 4, 8 and 12 h. After each spiking time period, an aliquot of 250 μL of spiking solution was analyzed by HPLC. The depletion of the concentration in the spiking solution was considered as the analyte absorption in the spiked tooth.

The spiked teeth were immersed in 20-mL glass vials sealed with aluminum caps with 3.0 mm thick PTFE septa, containing 1 M NaOH without headspace to extract all analytes in one step. The kinetic of extraction of BTEX and metabolites was studied in an ultrasonic bath at 4, 8 and 10 h. An aliquot of 250 μL for each extraction time was analyzed by HPLC.

2.2. SPME procedure

An 85 μm CAR/PDMS fiber (Supelco, Madrid, Spain) was used for BTEX extraction and an 85 μm PA fiber (Supelco) was used to extract the phenols and aromatic acids. Both fibers were preconditioned during 30 min at 30 °C with MeOH or HPLC mobile phase (10 mM Na_3PO_4:MeOH, 80:20 *v*/v), respectively. Spiked solutions containing 10 μg/mL BTEX, 50 μg/mL phenols and 50 μg/mL aromatic acids were prepared from 2500 μg/mL stock solutions by diluting the corresponding volume in 10 mL of 1 M NaOH solution. These solutions were placed in the 20-mL glass vials and immediately sealed with aluminum caps with 3.0 mm thick PTFE septa. Vials were warmed into a 30 °C water bath and agitated (500 rpm, magnetic stirrer). Once equilibrium was reached (10 min), three consecutive extractions were performed to each spiked solution. Firstly, BTEX were extracted from the headspace with CAR/PDMS fiber. Once the equilibrium was reached, the fiber was withdrawn from the headspace and desorbed by immersing it in MeOH (250 μL-vial without headspace). Secondly, the vial containing the spiked solution was brought to pH 2 (HCl, JTBaker, Tarragona, Spain) and saturated with NaCl (300 g/L); then volatile phenols were extracted from the headspace with PA fiber. When equilibrium was reached, the PA fiber was desorbed in mobile phase (250 μL-vial without headspace). Thirdly, non-volatile phenols and aromatic acids were extracted by immersing the PA fiber into the solutions. Analytes were desorbed in mobile phase (250 μL-vial without headspace).After each extraction-desorption operation was completed, the fiber was cleaned by immersing it in methanol or the HPLC mobile phase for 10 to 20 min and dried in the air for 1 min (blanks were analyzed to ensure the good performance of the cleaning).

For HS-SPME-GC/MS, desorption steps were carried out in the GC injector port. Extraction time (from 10 to 75 min) and desorption time (from 10 to 40 min) were studied to ensure effective SPME extraction of BTEX and metabolites. Solution was freshly prepared before each SPME procedure. A flow chart of the SPME procedure is shown in Fig. 1.

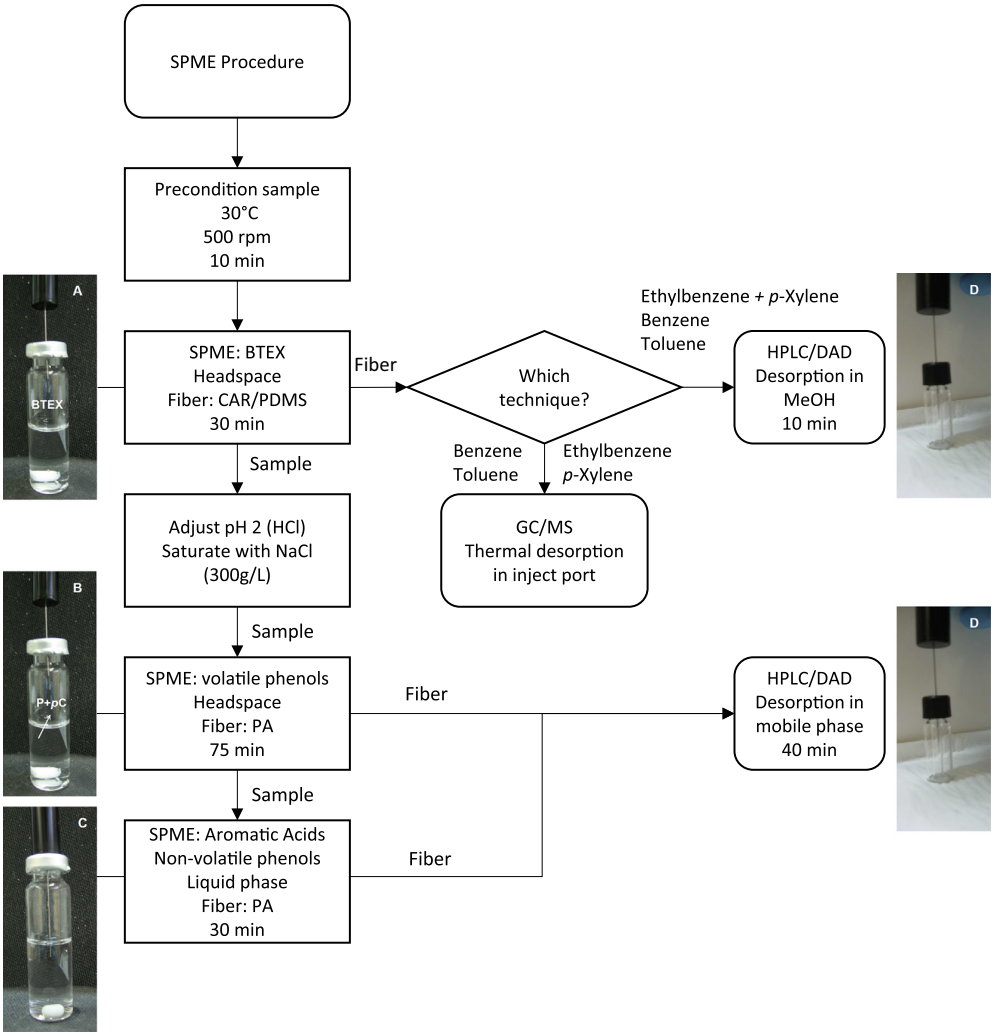

Fig. 1. Flowchart of the SPME procedure with the position of the fiber for (a) BTEX HS-SPME, phenols HS-SPME, (c) phenols and aromatic acids and (d) desorption process.

2.3. Chromatographic conditions

A high-pressure liquid chromatograph equipped with an autosampler AS3000, gradient pumps P4000 and UV detector UV6000LP, with measurement facility at 192–800 nm, equipped with ChromQuest Software (Thermo-Fisher, USA) was used. The separation was performed on an Alltech C-18 cartridge column (250 × 4.6 mm, 10 μm) with a mixture of 10 mM Na_3PO_4 $12H_2O$ (pH adjusted to 7.0 with concentrated orthophosphoric acid) as mobile phase A, and methanol as mobile phase B. The elution gradient was 20% B (0–2 min), 20–60% B (2–6 min), 60–80% B (6–10 min), 80% B (10–18 min) at a 1 mL/min flow-rate and 50 °C of temperature. The sample volume was 100 μL and the wavelength used for peaks integration was 260 nm for BTEX and phenols, and 220 nm for aromatic acids. As ethylbenzene was not well separated from the *p*-xylene isomer in the chromatogram; both compounds were analyzed as a mix peak in this case. The calibration curves were identified by comparison of the retention times and UV spectrums of the BTEX and metabolites standards. Quantitative analyses were based on external standards BTEX and metabolites (2.5, 5, 10, 25 μg/mL), analyzed by triplicate before each sample batch. Blanks from extractions were prepared for each calibration and sample batchs. Samples were analyzed by triplicate.

A Thermo Scientific TRACE GC Ultra™ equipped with a split/splitless injector and coupled to a Thermo Scientific DSQ™ II single quadrupole GC/MS system (Barcelona, Spain) was used for BTEX study. Analytes were separated with a SGE Analytical Science (Barcelona, Spain)

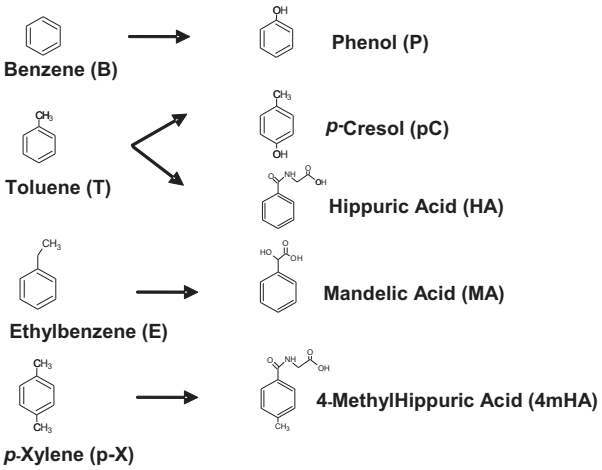

Fig. 2. Molecular structures of BTEX and five metabolites.

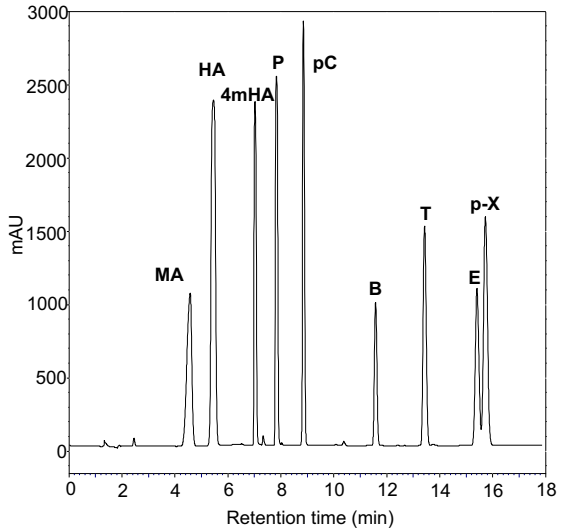

Fig. 3. HPLC chromatogram of BTEX, phenols and aromatic acids at 260 nm. Alltima C18 column, gradient of mobile phase 10 mM trisodium phosphate, pH 7 (20%) and methanol (80%) at 1 mL/min ($T = 50$ °C).

capillary column (30-m × 0.25-mm WCOT fused silica, 0.5-μm BP-5 stationary phase). The GC oven temperature program was as follows: 35 °C held 3 min, rate 5 °C/min to 90 °C, rate 100 °C/min to 250 °C, held 10 min, using helium at a constant flow rate of 1 mL/min. The injector was equipped with a 0.8-mm I.D. liner and injector temperature was maintained at 290 °C for the 85 μm CAR/PDMS fiber, with split-less mode at initial time followed by a 1:50 split ratio at 3 min (Grob method). Electron impact ionization (EI, 70 eV, temperature of ion source 120 °C) was used as ionization mode for BTEX. The calibration curves were identified by comparison of the retention times and MS spectrums of the BTEX standards. Quantitative analyses were based on internal standard (chlorobenzene, analyzed by triplicate before each sample batch. Samples were analyzed by triplicate.

2.4. Validation of SPME-HPLC and SPME-GC–MS method in spiked solutions

The spiked solutions were prepared from stock solution (2000 μg/mL) by appropriate dilutions of the standards in a 20 mL-vial containing 10 mL of 1 M NaOH solution for a concentration ranging from 2.5 to 25 μg/mL for HPLC and 2 to 20 μg/L for GC. The vials were immediately sealed with aluminum caps with 3.0 mm thick PTFE septa. For the SPME of each analyte, the optimized conditions of extraction and desorption time were used. Triplicate analyses and three extractions (until the complete extraction of the analytes) were performed. Calibration curves were constructed by plotting the peak area against the concentration of each analyte in the spiked solutions and were used for the calculation of linearity, limit of detection (LOD) and precision of all analytes. The estimation of LODs was based on the lowest detectable peak with a signal-to-noise ratio of three (Vial and Jardy, 1999) and the distribution constants (K_d) of BTEX, phenols and aromatic acids were calculated as described by van Door et al. (van Doorn et al., 1998).

3. Results and discussion

3.1. Chromatographic separation by HPLC

BTEX, phenols and aromatic acids are compounds with different substitutions in the aromatic ring (Fig. 2). The analysis of BTEX and phenols is fundamentally carried out by GC, while aromatic acids by HPLC. The analysis of phenols and aromatic acids by GC require a previous derivatization step to obtain a significantly better peak shape than the free molecules. To avoid a long time sample preparation, the direct analysis of these nine compounds in the HPLC system was carried out. The use of different HPLC chromatographic conditions permitted to found the best chromatographic separation for the nine compounds. The chromatography conditions were found on the basis of the following experiments.

UV absorption spectrums of BTEX and metabolites were obtained. The aromatic acids absorb at 210, 220 and 230 nm, respectively, while phenols and BTEX absorb around 260 nm. The peak integration of phenols and BTEX at 220 nm is affected by the presence of methanol in the mobile phase (>50%) due to the strong absorbance of this solvent. To avoid this problem and to eliminate the possible blank effect at low wavelength, the chromatographic separation was established at 260 nm for BTEX and phenols, and at 220 nm for aromatic acids.

As shown in the Fig. 3, the column permitted an adequate separation in 18 min of analysis with the exception of ethylbenzene and *p*-xylene isomers, which were not well separated in the chromatogram of samples. The optimal separation was found at 50 °C and the samples were dissolved in mobile phase to obtain an adequate resolution of the peaks. The resolution of aromatic acids was rising with increasing pH in the range 3–7. The best combination of overall performance was achieved with mobile phase pH 7 containing methanol as organic modifier due to the presence of —OH groups in the structure of these compounds. This is the first report about the simultaneous separation of these three families of aromatic compounds by HPLC.

3.2. Spiking and extraction procedures

Spiking procedure of tooth is a difficult step in the development of analytical method due to the characteristic of this material. The optimization of the spiking procedure was carried by triplicate to know the

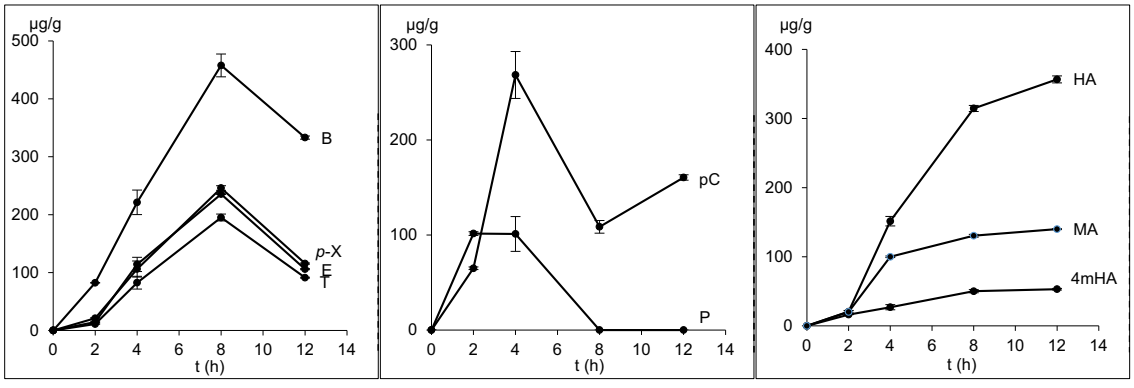

Fig. 4. Kinetic of BTEX and metabolites absorption in tooth.

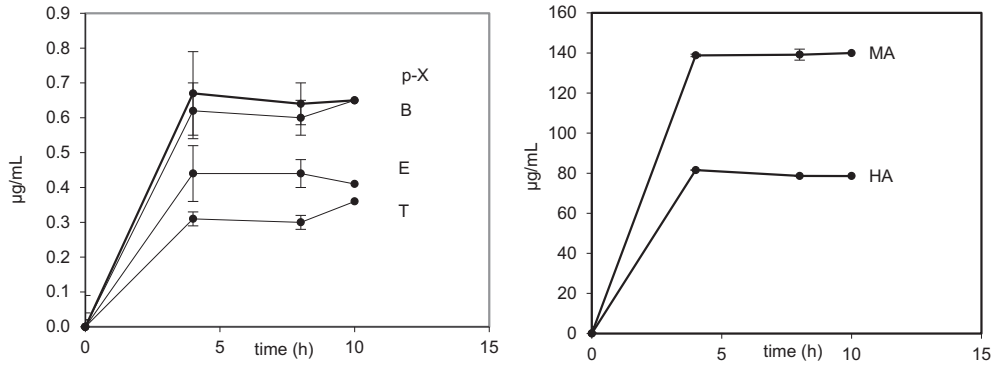

Fig. 5. Influence of the extraction time on the extraction of BTEX, phenols and aromatic acids from spiked teeth with 1 M NaOH in ultrasonic bath.

kinetic of absorption of BTEX and metabolites in tooth. The standard solution (made in methanol/water, 1:1) and ultrasonic bath were used for the spiking optimization. The results are shown in the graphics of concentration of compounds absorbed in the tooth (µg/g) *versus* time of absorption (Fig. 4).

The highest absorption for BTEX, phenols and aromatic acids was at 8, 4 and 12 h, respectively. In the graphics of the kinetic of BTEX and phenols absorption is shown an unusual behavior after 8 and 4 h, respectively. These results may be caused either the denaturalization of the tooth during the long time ultrasonication with the further BTEX and phenols release from the tooth to the solution or the migration of these volatile compounds to the headspace formed in the 20-mL vial after a long spiking time. The optimal time to obtain the spiked tooth was 4 h for the nine compounds.

The extraction step was optimized with a solution of NaOH 1 M as extracting from spiked tooth to recover the BTEX and metabolites in one step. The extraction was investigated through the kinetic of extraction with NaOH 1 M in ultrasonic bath. Studies of BTEX and phenols in solid samples (soils) describe the extraction step with water (Ezquerro et al., 2004; Baciocchi et al., 2001). Moreover, the extraction of aromatic acids from soils with 1 M NaOH solution is described by Fan and Deng (Fan and Deng, 2002). Because of the different polarity and acidity of these nine compounds, 1 M NaOH solution was selected to extract all mixture from the spiked teeth. The kinetic of extraction of BTEX and metabolites with 1 M NaOH is shown in Fig. 5. Phenols and 4-methylhippuric acid were not determined due to the matrix effect. Four hours was the optimal time extraction selected to extract the BTEX and metabolites in one-step extraction from spiked tooth.

3.3. Selection of SPME conditions in spiked solutions

3.3.1. General conditions

SPME is an equilibrium extraction method based on partition between the concentration of analytes in a sample and that in the solid-phase fiber coating (Arthur and Pawliszyn, 1990). The SPME efficiency of analytes from samples could be affected by type of fiber, sample matrix, temperature, absorption time, desorption time, and other factors.

The selection of an appropriate coating is important for the SPME method. Low molecular weight or volatile compounds as BTEX usually require a coated PDMS fiber. PDMS fiber is relatively non-polar and can easily extract the non-polar substituted benzenes. Its behavior is similar to that of hexane in a liquid-liquid extraction (Buchholz and Pawliszyn, 1994). It is also known that CAR/PDMS fiber is the best coating for simultaneous extraction of low-concentration BTEX from headspace (Ezquerro et al., 2004) or by immersion in liquid samples, that contain trace-level of BTEX (Lee et al., 2007). For phenols and aromatic acids, a more selective polar coating is required for their extraction, where the PA coating clearly shows better sensitivity than other fibers (van Doorn et al., 1998; Barták and Čáp, 1997; Huang et al., 1997).

During the extraction step with 10 mL of 1 M NaOH solution from the tooth by ultrasonication, the volatile and non-soluble BTEX easily migrate from basic solution to the headspace of the 20-mL vial. This step allowed the separation of BTEX in the headspace to extract by HS-SPME, whereas the phenols and aromatic acids were kept in the aqueous solution in their dissociated form. When the pH is lowered up to pH 2 with the addition of HCl, their acid-base equilibrium shifts significantly toward the neutral form, which has a greater affinity for the fiber, thereby increasing the amount extracted. The presence of the NaCl (300 g/L) decreases their solubility in the aqueous solution and forces more of these analytes into the fiber by salting-out effect. Although a stir bar accelerates the mass transfer of volatile and semi-volatile analytes through the aqueous matrix. The combination of both acidification and salting-out effects permitted the migration of phenols to headspace and the HS-SPME extraction of them (Buchholz and Pawliszyn, 1994; Barták and Čáp, 1997). After that, the SPME of aromatic acids by immersion of the fiber in the aqueous solution was carried out. These consecutively SPME steps with three number of

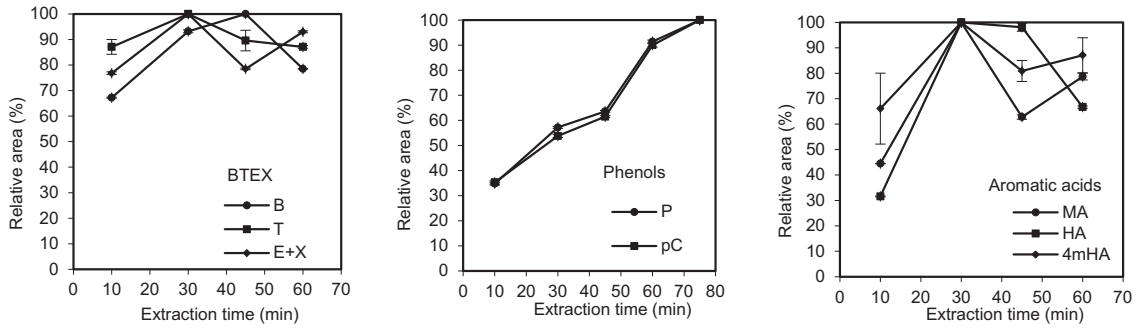

Fig. 6. Influence of the absorption time on the SPME of BTEX, phenols and aromatic acids in spiked solutions at 30 °C using PDMS and PA fibers and 30 min desorption time.

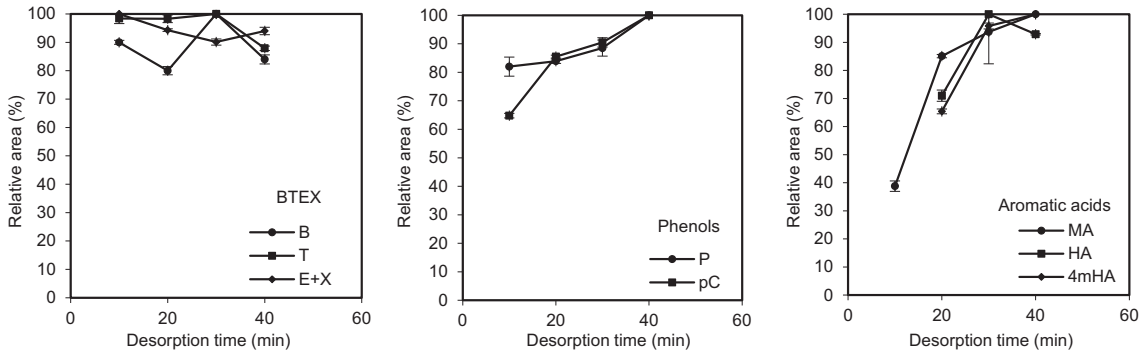

Fig. 7. Influence of the desorption time on the SPME of BTEX, phenols and aromatic acids in spiked solutions at 30 °C using 85 μm PDMS and PA fibers and previous optimized absorption times (30, 75 and 30 min, respectively).

extraction (exhaustive extraction of each analyte) from the same sample allowed the specific extraction of each group of analytes without interferences between them.

3.3.2. Absorption and desorption times

The time to reach absorption equilibrium determines the maximum amount of analyte that can be extracted by the fiber. The extraction time was the first SPME variable studied in spiked solutions through the consecutively SPME of each group of analytes as described above. Fig. 6 shows the extraction curves obtained for BTEX, phenols and aromatic acids in spiked solution for extraction times ranged to 10–75 min. The maximums were observed at 30, 75 and 30 min, respectively. The extraction times were selected as the time it takes for the fiber to absorb near 100% of the equilibrium extractable mass of analytes. This extraction time is in agreement with a previous report for these analytes in spiked solutions (Ezquerro et al., 2004; Fan and Deng, 2002; Huang et al., 1997). For BTEX, the amounts increased rapidly within the first 10 min and tended to reach constant values (>70% of the extracted analytes) after 20 min, whereas the equilibrium was reached within 60 min (>90% of the extracted analytes) for phenols. The slower equilibration times with PA coating for phenols extraction is due to two main causes. First, the relatively low equilibrium vapor pressure of phenols provokes a slow transfer from the aqueous layer through the headspace to the fiber (Zhang and Pawliszyn, 1993). Secondly, the nature of the PA fiber as a polymeric solid do not permit an easily diffusion into and through the coating (Buchholz and Pawliszyn, 1994). However, the aromatic acids behavior was different after to reach the maximum extraction at 30 min, where the values start to be unstable. It may be caused for the migration of analytes after the equilibrium from the coating layer of the polar PA fiber to aqueous phase.

Desorption experiments were carried out for 10, 20, 30 and 40 min. Because of the low solubility of BTEX in aqueous solutions, methanol and HPLC mobile phase solvents were used to find the optimal solvent for desorption of CAR/PDMS fiber with extracted BTEX. The results obtained permitted the selection of methanol as the best solvent for desorption of BTEX from the fiber in all desorption time range. The desorption-time profiles for all analytes are shown in Fig. 7 A selection was made by choosing 10, 40 and 30 min as optimized desorption time for BTEX, phenols and aromatic acids, respectively. The BTEX desorption process occurred rapidly for the first 10 min, and >80% of the extracted analytes were desorbed in methanol. Also, for phenols, after 20 min of desorption, >80% of the extracted analytes were desorbed. For aromatic acids, the maximum was observed at 30 min, but the equilibrium was reached at 40 min.

3.4. SPME method validation

The linear ranges, the linearity, the slope and intercept with their standard deviations, the correlation coefficients, the LODs, the precision and the distribution constant (Kd) were calculated when the optimum conditions for SPME-HPLC and SPME-GC/MS procedures were established. For BTEX, the method validation was carried out by HS-SPME followed by HPLC and GC/MS separation techniques (Table 2 and Table 3, respectively). Some GC/MS parameters set were in accordance with Lee et al. (Lee et al., 2007), such as the desorption temperature, desorption time and GC oven temperature program. For phenols, the method validation was established by SPME-HPLC from both the immersion of the fiber in the headspace and in the spiking solution (Table 2).

Table 2
Linear range, limit of detection (LOD) and precision of BTEX, phenols and aromatic acids in spiked solutions by SPME-HPLC[a].

Compound	Studied range (μg/mL)	Linear range (μg/mL)	Slope	Intercept	r^2	LOD[b] (ng/mL)	RSD (%) (mass level, μg/mL)	Kd
Mandelic acid	0–24.0	2.9–24.0	0.6 ± 0.02	−0.70 ± 0.03	0.998	33.3	0.5 (5)	6
Hippuric acid	0–24.0	3.0–24.0	21.2 ± 0.5	−2.0 ± 0.7	0.999	1.8	1.0 (2.5)	98
4-Methylhippuric acid	0–26.0	3.0–26.0	32 ± 2	−5 ± 3	0.994	6.1	1.0 (25)	207
Phenol (HS-SPME)	0–26.0	2.7–26.0	12.4 ± 0.3	−2.6 ± 0.5	0.998	5.5	1.0 (25)	344
Phenol (SPME)			7.8 ± 0.3	−0.7 ± 0.5	0.996	4.3	0.5 (5)	310
p-Cresol (HS-SPME)	0–25.0	2.4–25.0	9.23 ± 0.03	−0.74 ± 0.04	1	0.8	0.2 (50)	687
p-Cresol (SPME)			7.82 ± 0.07	−0.61 ± 0.09	0.999	4.9	0.7 (5)	319
Benzene	0–25.0	2.5–25.0	1.10 ± 0.06	0.56 ± 0.08	0.994	7.5	0.1 (100)	264
Toluene	0–25.0	2.5–25.0	7.9 ± 0.4	5.2 ± 0.6	0.994	1.1	0.2 (100)	916
Ethylbenzene + *p*-xylene	0–25.0	2.5–25.0	48 ± 1	23 ± 2	0.998	0.2	0.04 (100)	2468

[a] Three replicates and three consecutive SPME for each group of analytes: BTEX by HS-SPME-GC/MS method, phenols by both HS-SPME-HPLC and SPME-HPLC methods, and acids by SPME-HPLC method.
[b] Determined by signal-to-noise ratio method.

Table 3
Linear range, limit of detection (LOD) and precision of BTEX in spiked solutions by HS-SPME-GC/MS[a].

Compound	Studied range (ng/mL)	Linear range (ng/mL)	Slope	Intercept	r^2	LOD[b] (pg/mL)	RSD (%) (mass level, ng/mL)	Kd
Benzene	0–200	20–200	0.019 ± 0.001	−0.3 ± 0.1	0.987	0.6	3.6 (20)	10,091
Toluene	0–200	20–200	0.034 ± 0.003	−0.3 ± 0.1	0.990	0.8	0.8 (20)	9849
Ethylbenzene	0–200	20–200	0.069 ± 0.009	−2 ± 1	0.984	0.9	1.6 (20)	5624
p-Xylene	0–200	20–200	0.071 ± 0.005	−1.4 ± 0.7	0.996	0.9	2.4 (20)	5459

[a] For three replicates and one SPME.
[b] Determined by signal-to-noise ratio method.

The linearity of SPME-HPLC method was studied by extracting standard solutions spiked 2.5, 5, 10, 25 μg/mL BTEX and metabolites, using the optimum absorption and desorption times. The r^2 were above 0.994 for all analytes. Triplicate analysis with three SPME extractions for each spiked sample was performed with a precision lower than 15%. LODs were 2–333 ng in spiking solution (0.2–33.3 ng/mL). The Kd values were determined for the first time by SPME-HPLC method and all Kd values were higher than the reported by SPME-GC (van Doorn et al., 1998; Buchholz and Pawliszyn, 1994; Barták and Čáp, 1997; Arthur et al., 1992; Langenfeld et al., 1996; Nardi, 2003). These values demonstrate the sensitivity of SPME-HPLC method studied. The features of both HS-SPME and SPME methods for phenols analysis showed similar validation parameters.

To obtain a good separation of the isomers *p*-xylene and ethylbenzene, the validation of HS-SPME-GC/MS method was carried out for BTEX (Table 3). The linearity was examined from standard solutions containing 2, 5, 10, 15 and 20 ng/mL of BTEX. It is well known that the GC/MS technique is more sensitive than the HPLC technique. For that reason, the LODs (0.6–0.9 pg/mL) and Kd values obtained by this technique were better than the HS-SPME-HPLC method. The r^2 and precision were quite similar for the BTEX analysis by both SPME methods.

3.5. Application in human teeth

The proposed SPME method was used to quantify BTEX and metabolites in human teeth. The selected HS-SPME-GC/MS, HS-SPME-HPLC and SPME-HPLC methods for the consecutive analysis of BTEX, phenols and aromatic acids, respectively, were operated under the optimum conditions. Consecutive SPME were performed for all analytes from the same tooth sample. The results in Table 4 show that BTEX was present in almost all human teeth. The major content of BTEX was obtained for teeth samples 2 and 7, with >1 μg/g. These teeth samples belong to a smoking old man and non-smoking young man. This result shows that the presence of BTEX in these human teeth samples is not necessary caused by tobacco smoke and might be related by some exposure of BTEX. Another interesting sample was tooth 3 with an important content of ethylbenzene and *p*-xylene (near 1 μg/g). This tooth sample belongs to a young woman which works in solvent industry. In relation of phenols, the quantification of both phenol and *p*-cresol was similar for all human teeth in the range 36–99 μg/g. It is well known that phenols appear in a common human diet. For that reason, the similar results obtained for phenols in all teeth samples could be not an indicator of BTEX exposure.

The results indicated the suitability of the consecutive SPME method for analyzing trace BTEX and phenols in human teeth, but for the other metabolites, such as mandelic and hippuric acids were not identified.

4. Conclusions

The present study of one-step simple extraction of BTEX and metabolites (phenols and aromatic acids) from tooth sample with NaOH solution followed by a specific and consecutive SPME-GC/MS or SPME-HPLC procedure for each analyte indicate an adequate precision of the method with a good linearity for analyzing this compounds in human teeth. Better sensitivity was obtained by HS-SPME combined with GC/MS in the analysis of BTEX. In contrast to many advantages of SPME (simple, rapid, sensitive, accurate, solvent-free, no sample pre-treatment), it showed that in the case of SPME-HPLC procedure, the LODs were a little high with respect of other methods. Therefore, as far as the determination of non-volatile (mandelic and hippuric acids) compounds by consecutive SPME extraction is concerned, it is necessary to find another procedure with a high sensitivity to obtain lower LODs to determine these metabolites in human teeth. This work may contribute to ascertain the evolution of some environmental contaminants on human dental tissues.

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Table 4
BTEX and metabolites determination by SPME-GC/MS and SPME-HPLC methods in human teeth as biomarkers.

Sample teeth	Age (years)	Smoker	Profession or work place	Amount of BTEX (ng/g) and metabolites (μg/g)[a]								
				B	T	E	X	P[a]	*p*C[a]	MA	HA	4mHA
1	28	Yes	Painting	26	18	25	25	42	99	–	–	–
2	71	Yes	Mechanic	1386	1967	1519	1883	54	69	–	–	–
3	32	No	Solvent industry	76	200	958	728	42	36	–	–	–
4	56	Yes	Construction company	37	23	–	–	51	36	–	–	–
5	59	No	Construction company	126	95	72	84	84	69	–	–	–
6	24	No	Construction company	51	19	8	12	48	69	–	–	–
7	29	No	Construction company	1085	1640	1665	1978	54	72	–	–	–
8	42	No	Construction company	118	42	10	10	45	75	–	–	–
9	71	Yes	Coffee factory	19	15	7	10	51	36	–	–	–
10	35	No	Pharmaceutical Ind.	78	23	12	18	72	57	–	–	–

[a] Three replicates and three consecutive SPME for each group of analytes: BTEX by HS-SPME-GC/MS method, phenols by both HS-SPME-HPLC and SPME-HPLC methods, and acids by SPME-HPLC method.

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