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In vivo solid phase microextraction sampling of human saliva for non-invasive and on-site monitoring

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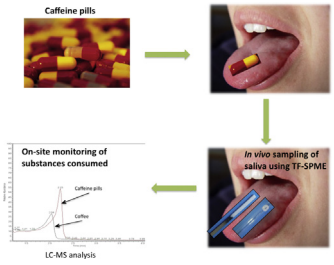
HIGHLIGHTS

- 5 min *in vivo* SPME sampling of saliva offers unbiased on-site monitoring.
- TF-SPME–LC–MS provides fast determination of prohibited substances in saliva.
- TF-SPME provides very good sample clean-up, preventing matrix effect.
- The TF-SPME–LC–MS method offers enough sensitivity to detect steroids in saliva.

GRAPHICAL ABSTRACT



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ABSTRACT

On-site sample preparation is an analytical approach based on direct sampling from the system under investigation. It has the advantage of combining sampling and sample preparation into a single step, thus generally is fast, minimizes the potential sources of error and eliminates the risks for analytes instability. For such analysis solid phase microextraction in thin film geometry (TF-SPME) can provide robust and convenient *in vivo* sampling, offering in the same time faster analysis and higher extraction recovery (*i.e.*, better sensitivity) due to large surface to volume ratio.

In this study, TF-SPME in coated blade and membrane formats with a single extraction phase were used for *in vivo* and *ex vivo* saliva extraction and separation by LC and GC, respectively. Due to applicability for wide range of polarity of analytes as well as thermal and solvent stability during the desorption, hydrophilic lipophilic balanced particles (HLB) were chosen as extraction phase and used for fast (5 min) *in vivo* and *ex vivo* sampling. The results of metabolomic profiling of the saliva are indicating that even 5 min *in vivo* sampling using TF-SPME followed by GC and LC analyses provides complementary coverage of wide range of analytes with different physical and chemical properties. To demonstrate the applicability of the method for doping analyses, the SPME–LC–MS/MS method was validated for simultaneous quantification of 49 prohibited substances with limit of quantification (LOQ) ranging between 0.004 and 0.98 ng mL^{-1}. Moreover, the method was also validated and successfully applied for determination of endogenous steroids in saliva where the concentrations of the analytes are substantially low. The developed assay offers fast and reliable multiresidue analysis of saliva as an attractive alternative to the standard analysis methods.

1. Introduction

There is a growing interest for saliva as alternative specimen for drugs detection in forensic and clinical chemistry [1,2]. Saliva sample collection is easy, non-invasive and does not require any

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special training compared to blood sampling. In addition, for doping control or drug abuse, saliva is available at any time and sample can be collected at the public view to prevent adulteration or sample substitution. Saliva is the direct filtration of blood and contains approximately 99% of water with electrolytes, mucus, proteins and small molecules [3]. Excretion of drugs into saliva is mainly controlled by passive diffusion for hydrophobic compounds and by ultrafiltration for hydrophilic compounds with a low molecular weight (<1900) [1,4]. The basicity of the drug is also an important property, which controlled the transfer from the blood. Higher concentrations of basic compounds are found in saliva due to their ionization associated with the transfer from blood (pH 7.4) to saliva (pH 6) [1].

Contrary to urine, saliva contains only a small amount of biotransformation metabolites (*e.g.*, conjugated metabolites), because these metabolites are poorly excreted into saliva due to an increase in molecular weight, acidity and hydrophilicity [1,4]. Therefore, saliva offers the possibility to measure the free fraction of the drug, which is the biologically active form. The major disadvantages of saliva are the very low concentrations of xenobiotics (nanomolar range) and the influence of the salivary flow rate on concentrations of hydrophilic compounds, since these compounds are excreted by ultrafiltration [1,2,4]. Saliva collection can be easily performed by spitting, draining, chewing an inert material or wiping oral cavity using commercial devices such as Salivette® or Drugwipe®. The main issue with some commercial devices is the incomplete recovery of the drug due to their absorption/adsorption on the collection devices, which can underestimate drugs' concentration [2,5].

Solid-phase microextraction (SPME) has been recently introduced as a simple, fast and sensitive sample preparation technique for monitoring xenobiotics in various biological fluids [6,7]. The extraction of analyte is achieved using a fused-silica or stainless steel support coated with a extraction phase exposed to the sample. The non selectivity of the fiber provides the extraction and the pre-concentration of a wide range of small molecules, which are further desorbed either thermally in the GC injector port for GC–MS analysis or in a small volume of organic solvent (1 mL or less) for LC–MS analysis, according to their physical and chemical properties. Recent results have shown that SPME can simultaneously extract more than 400 metabolites from human saliva [8]. Recently, a new geometry of SPME, thin-film microextraction (TF-SPME), was introduced providing higher sensitivity and shortest extraction time due the high surface area-to-volume ratio, and high-throughput sample preparation when combined with the automated Concept 96-blade system [9,10]. The small size and the biocompatibility of the SPME coating opens the way to *in vivo* sampling in direct immersion extraction mode [7,11,12].

In the present study, we evaluated the ability of *in vivo* SPME sampling of saliva, in combination with LC-high resolution MS and GC–MS methods for unbiased retrospective analysis of substances consumed, and developed and validated a SPME–LC–MS/MS for simultaneous quantification of 49 prohibited substances and endogenous steroids in saliva, in order to demonstrate the analytical figures of merits of the methods.

2. Materials and methods

2.1. Thin-film microextraction coatings preparation

Hydrophilic lipophilic balanced (HLB) particles (60 μm, average particle diameter) were used as a primary extraction phase in two different geometries, namely, thin-film coated blades and thin film membranes. In addition, as indicated in the related sections, C18 particles (5 μm, average particle diameter) coated blades were also utilized as extraction phase for some parts of the study. The thin film coated blades with coating thicknesses of *ca* 165 μm and 60 μm for HLB and C18 particles, respectively, were prepared as described by Mirnaghi et al. [13] by immobilization of particles on the surface of stainless steel blade. Immobilization of particles was accomplished by the aid of polyacrylonitrile (PAN) solution, which was acting as glue in the process. The salivary extract in the thin film coated blades was desorbed by an appropriate solvent and used for LC–MS analysis.

On the other hand, for analysis of GC–MS detectable analytes from salivary extract, HLB particles were immobilized on PDMS membrane (HLB–PDMS), thus thermally stable extraction phase with the same primary extractive functionality was obtained. HLB–PDMS membrane was prepared as follows; 0.2 g of HLB particles was dispersed in 1 mL of hexane by a preliminary 1 min vortex mixing, which was followed by 30 min of sonication. Resulting mixture was merged with 1.0 g of PDMS base (Dow Corning) and mixed thoroughly by 1 min vortex followed by 30 min sonication.

In order to start the cross-linking and condensation of PDMS base to form an elastomer a curing reagent should be added to the mixture in a ratio of 1:10 (w:w, curing agent: PDMS base). The cross linking process is initiated and accelerated by a thermal curing. In order to ensure that the cross-linking is not initiated when the curing reagent is added to HLB–PDMS mixture, the mixture was cooled for 10 min in a fridge. After cooling, 0.1 g of curing reagent was added and vortexed for 1 min. To obtain the final mixture with appropriate viscosity for thin film application, hexane was evaporated by blowing N_2 directly into the mixture until constant weight of the vial was obtained (±10 mg). The resulting mixture was spread in to a thin film using Elcometer 4340 automatic film applicator (Elcometer Inc., Manchester, UK).

The resulting film was cured in a vacuum oven initially at 80 °C for 5 h then at 120 °C for additional 5 h, all under N_2. After the curing step, membranes with the thickness of *ca.* 145 μm was obtained, the image of a typical HLB–PDMS membrane is shown in Supplementary information Fig. S-1. Circular membranes with 6 mm of diameter were cut from the bulk membrane and were conditioned in a thermal conditioner unit (GERSTEL GmbH, Mullheim, GE), initially for 5 h at 200 °C and then at 250 °C for additional 5 h, all under N_2. The prepared membranes were further conditioned four times for 1 h in twister desorption unit of GC–MS prior to use.

2.2. Metabolites profiling of human saliva

2.2.1. Study design, sample collection and sample preparation

One goal of this study was to investigate the efficiency of *in vivo* SPME sampling of saliva, compared to *ex vivo* sampling, for retrospective detection of substances consumed. Two participants were involved in this study: one 32-year-old male and one 37-year-old female. The volunteers consumed different substances the days of the sampling: 100 mg (coffee) and 200 mg of caffeine (pills), 6 mg of benzocaine (sore-throat medication) and 325 mg of acetaminophen (Tylenol). All sampling were conducted at least 30 min after consumption of substances and at least 4 h after using any oral hygiene products. All experiments were conducted using PAN–HLB coated blades and PDMS–HLB membranes. Before sampling, all probes (TF-SPME blades and membranes) were sterilized by direct immersion for 15 min in 70% ethanol solution. Then, four compounds (eugenol, menthol, linalool and vanillin) were preloaded for 30 min on the probes from direct extraction in aqueous standard solution (10 ng mL^{-1}). These compounds were used as calibrants, in order to correct for differences in agitation conditions, sample volume and sample temperature between *in vivo* and *ex vivo* SPME sampling, and between individuals. After preloading, all probes were kept at −80 °C before sampling to avoid loss of calibrants. The sampling procedure was as follows: