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ORIGINAL ARTICLE

Preconcentration of organic solutes in urine by bubble bursting

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Abstract

Introduction The chemical sensitivity of urine metabolomics analysis is greatly compromised due to the large amounts of inorganic salts in urine (NaCl, KCl), which are detrimental to analytical instrumentation, e.g. chromatographic columns or mass spectrometers. Traditional desalting approaches applied to urine pretreatment suffer from the chemical losses, which reduce the information depth of analysis.

Objectives We aimed to test a simple approach for the simultaneous preconcentration and desalting of organic solutes in urine based on the collection of induced bursting bubble aerosols above the surface of urine samples.

Method Bursting bubbles were generated at ambient conditions by feeding gas through an air diffuser at the bottom of diluted (200 times in ultrapure water) urine solution (50–500 mL). Collected aerosols were analyzed by the direct-infusion electrospray ionization mass spectrometry (ESI–MS).

Results The simultaneous preconcentration (ca. 6–12 fold) and desalting (ca. six–tenfold) of organic solutes in urine was achieved by the bursting bubble sample pretreatment, which allowed ca. three-times higher number of identified urine metabolites by high-resolution MS analysis. No chemical losses due to bubbling were observed. The increased degree of MS data clustering was demonstrated on the principal component analysis of data sets from the urine of healthy people and from the urine people with renal insufficiency. At least ten times higher sensitivity of trace drug detection in urine was demonstrated for clenbuterol and salbutamol.

Conclusion Our results indicate the high versatility of bubble bursting as a simple pretreatment approach to enhance the chemical depth and sensitivity of urine analysis. The approach could be attractive for personalized medicine as well as for the diagnostics of renal disorders of different etiology (diabetic nephropathy, chronic renal failure, transplant-associated complications, oncological disorders).

Graphical Abstract Urine desalting and preconcentration in bursting bubbles.

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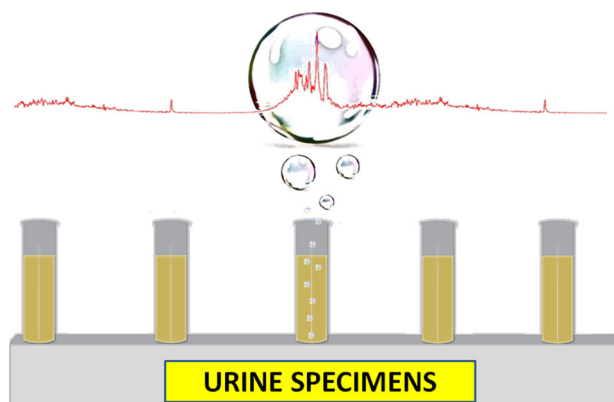
URINE SPECIMENS

1 Introduction

The relation of urine quality to physiological condition of humans has been recognized for many centuries. Because urine is sterile, easy and noninvasive to obtain in large volumes, urine analysis is nowadays widely employed in routine clinical diagnostic tests, e.g. to measure the level of urinary glucose, bilirubin, ketone bodies, nitrates, leukocyte esterase, specific gravity, hemoglobin, urobilinogen and protein, as well as to study a variety of renal conditions, such as bladder, ovarian and kidney diseases (Chen and Kim 2016; Turkoglu et al. 2016). However, despite the extensive chemical analysis of urine for more than 100 years many compounds in urine and their role in human metabolism still remain poorly understood due to the high chemical complexity of urine.

The current knowledge about urine chemicals is being mainly enhanced by ''global'' metabolomics approaches, such as high resolution nuclear magnetic resolution (NMR) spectroscopy (Bollard et al. 2005; Dieterle et al. 2011), high performance or ultra-high performance liquid chromatography (HPLC/UPLC) (Spagou et al. 2011; Meyer et al. 2011), liquid chromatography-tandem mass spectrometry (LC–MS/MS) (Kim et al. 2008; Wohlfarth et al. 2013) and gas chromatography-tandem mass spectrometry (GC–MS/MS) (Waldhier et al. 2011; Watanabe et al. 2011). Pretreatment of urine samples is usually required before the analysis. The major problem is the large amounts of inorganic salts (NaCl, KCl), which are detrimental to chromatographic columns and MS instrumentation. The use of traditional desalting approaches such as size-exclusion or ion-exchange chromatography results in significant losses of small and polar compounds, which are the main constituents of urine. Lyophilization and chemical extraction also suffer from chemical losses (Court et al. 2015). A popular sample pretreatment approach in urine analysis is the simple dilution (e.g., 100–1000 fold) of urine samples in water or organic solvent (Rosing et al. 1999; Kuligowski et al. 2014). However, sample dilution also results in the decreased visibility of chemicals present in urine at trace concentrations. Significant efforts are being made to develop more practical and versatile approaches for chemical preconcentration and purification relying on the recent advances in adsorbent materials (Liu et al. 2011; Ibrahim et al. 2015), microfluidics (Reinholt and Baeumner 2014) nanotechnology (Inglis et al. 2011), micro-extraction (Kailasa et al. 2014), crystallization (Gong et al. 2015), electrospray ionization (ESI) (Wei et al. 2013) and matrix-assisted laser desorption/ionization (MALDI) (Chen et al. 2010).

It has been earlier discovered that the concentration of organic solutes in bursting bubble aerosols above the sea surface is increased by more than two orders of magnitude relative to the bulk seawater whereas the chemical enrichment of inorganic solutes is limited to a few percent if occurs at all (McNeill et al. 2014; Schmitt-Kopplin et al. 2012). The effect has been attributed to the high surface activity of organic compounds, which allows them to gradually partition to the gas–liquid interface of rising bubbles in sea water (McNeill et al. 2014; Schmitt-Kopplin et al. 2012). Upon bubble bursting at the ocean surface these compounds are released into the air in tiny droplets. It has been recently demonstrated that the collection of induced bursting bubble aerosols above the surface of aqueous solutions could be used as a simple approach for the simultaneous preconcentration and desalting of organic solutes in aqueous solutions before mass spectrometry, chromatography, optical detection and other types of analysis (Chingin et al. 2016). Here we demonstrate the high suitability of the bubble bursting approach as an efficient pretreatment step to enhance the chemical depth and sensitivity of urine analysis.

2 Experimental

2.1 Bubble bursting

Bursting bubbles were generated at ambient conditions by feeding gas (1–100 kPa; 1–100 L/s) through an air diffuser at the bottom of diluted (200 times in ultrapure water) urine solution (50–500 mL). The 200-times dilution was chosen as a commonly used degree of dilution in direct-infusion ESI–MS. Weaker urine dilutions are usually avoided in order to avoid the chemical contamination of mass spectrometers and chromatographic columns. Diffusers with three different pore sizes were tested: 100, 1–10, 0.2–5 μm. The first diffuser was made in house by pulling ten fused silica capillaries with i.d. 100 μm through a rubber piece. The second diffuser was made from porous ceramics and was purchased from Shiyuan Appliances, Shenzhongshan, Guangdong, China. The third diffuser was made from porous ceramics and was purchased from Junfeng Trading Ltd., Ningbo, Zhejiang, China. The bursting bubble aerosol was collected using a slanted glass slide fixed above the liquid surface (Fig. 1). Typically, ca. 100 μL of aerosol was collected within 5–10 min from 500 mL bulk solution at the gas pressure of 10 kPa.

2.2 MS analysis

Collected aerosols and bulk solutions were analyzed in three technical replicates under identical experimental
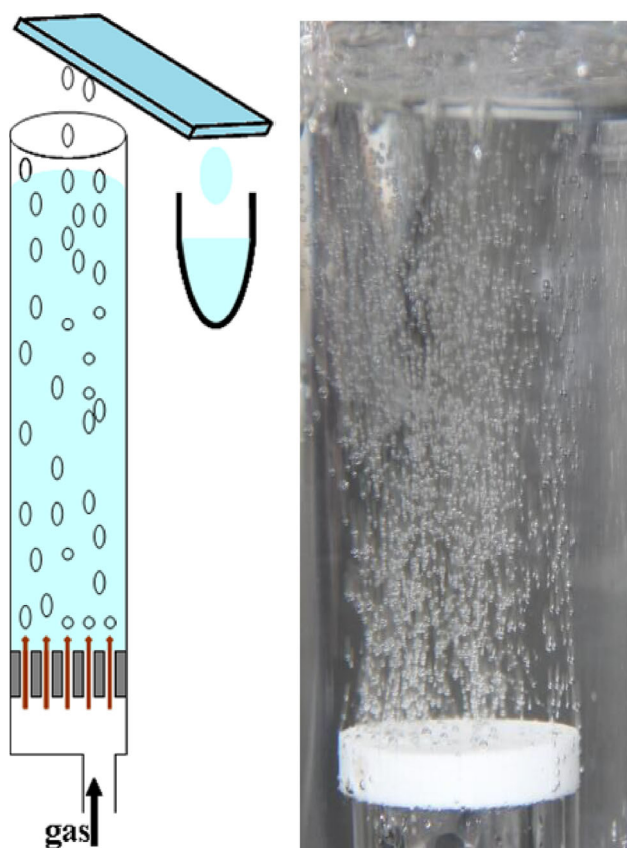
Fig. 1 Generation of bursting bubbles at ambient conditions by feeding gas through the porous surface at the bottom of the aqueous analyte solution

conditions by direct-infusion ESI–MS in positive ion detection mode using built-in loop injector (35 μL), unless specified otherwise. Experiments were done on LTQ-MS (Thermo Scientific, San Jose, CA) and FTICR-MS (Thermo Scientific, San Jose, CA) mass spectrometers. Chemical enrichment factors were derived as the intensity ratios of corresponding signals in the collected aerosol and bulk sample ($R = I^{bubble}/I^{bulk}$).

3 Results and discussion

3.1 Optimization of chemical enrichment by bubble bursting

The phenomenon of organics enrichment in bursting bubbles has been earlier described using a simple model of chemical partitioning (Chingin et al. 2016). Briefly, it was proposed that the chemical enrichment factor (R) for a particular solute was primarily determined by its surface activity and was further modulated by the presence of other solutes competing for the bubble surface. Before applying the method to urine analysis, here we performed a set of

model experiments to optimize experimental conditions for the higher preconcentration efficiency. Three gas diffusers with different porosity (100, 1–10, 0.2–5 μm) were tested to produce bubbles from the water solutions of arginine (Arg) and phenylalanine (Phe). The highest R-value was reproducibly obtained for the smallest pore size, other parameters being equal (Table S-1). It is suggested that smaller bubbles produced by smaller pores release less bulk water upon bursting, thus resulting in the higher organics concentration in the collected aerosols. R-values also grow with the bubble path in solution, as more organic solutes are being adsorbed by the bubble surface (Chingin et al. 2016). However, starting from a certain value (50–100 cm) further increase in the path length causes the decrease of R-values (Fig. 2; Table S-2). This phenomenon could be related to the gradual increase of bubble size due to bubble coalescence (Chingin et al. 2016). Further, the highest chemical enrichment was achieved at the lowest gas pressure needed to induce stable bubble bursting above the solution surface (Fig. 2). At higher pressures bubbles are produced at a higher rate, which increases the probability of bubble coalescence and associated bulk water transfer. Ultimately, the following bubble bursting parameters were selected for urine analysis (unless specified otherwise in the text): dilution factor = 200 times in water; sample volume = 500 mL; bottle bottom surface = 5 cm^2; solution level (height) = 100 cm; diffuser porosity = 0.2-5 μm; gas = nitrogen (N$_2$); gas pressure = 10 kPa; bubbling time = 5 min; distance from the solution surface to collection glass slide = 4 cm.

3.2 Bubble bursting for non-targeted metabolite screening

Three replicate urine samples from the same healthy human individual as well as three aerosol samples collected from those three replicates by bubble bursting were analyzed using direct-infusion ESI-FTICR-MS under identical conditions. MS signals with signal-to-noise ratio (SNR) >5 were searched across the global metabolite MS database from the Scripps Center for Metabolomics (METLIN: https://metlin.scripps.edu/metabo_search_alt2.php) with 1 ppm mass tolerance. The search tolerance of 1 ppm was chosen to accord with the ca. 0.5 ppm accuracy of FTICR-MS detection. The found metabolites were then additionally searched across the library of urine metabolites from the Human Metabolome Database (HMDB: http://www.hmdb.ca/) to refine multi-compound matches, i.e. MS signals with more than one metabolite found by METLIN. This is a common metabolite searching approach in urine metabolomics (Bouatra et al. 2013; Smith et al. 2005; Tautenhahn et al. 2012). Following this approach, each MS signal was ultimately put into unique correlation with no
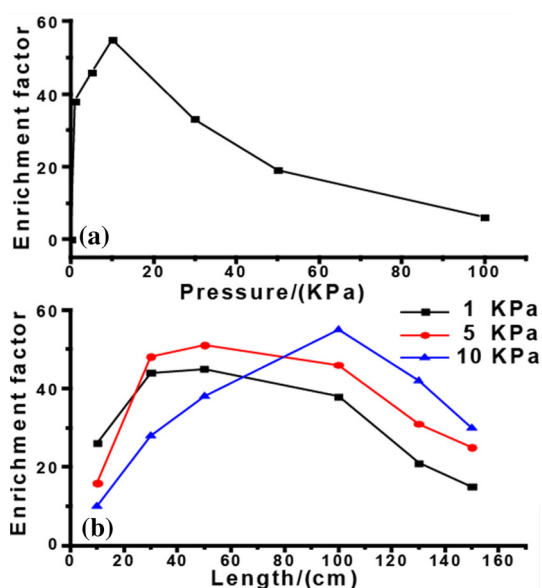
Fig. 2 The dependence of signal enrichment ($R = I^{bubble}/I^{bulk}$) for aqueous arginine (Arg, 20 μM) on gas pressure (**a**) and bubble path length (**b**). Bubbles were produced using air diffuser with porosity 0.2–5 μm. The bubble path in the top panel was 100 cm

more than one metabolite (Table S-3). The identity for some of the metabolites was further confirmed by reference MS/MS analysis of standard compounds (Table S-3). In total, independent analysis of three bulk urine samples returned 57, 59 and 60 urine metabolites, 49 of which were found in each sample (Fig. 3a). The analysis of three collected aerosol samples returned 150, 153 and 155 urine metabolites, 133 of which were found in each sample (Fig. 3b). The metabolites found in the bulk solution were also entirely present in the collected aerosol (Fig. 3c). Indeed, the ultimate number of identified metabolites depends on many experimental parameters, such as the sensitivity of mass analyzer used for ion detection, ionization efficiency, degree of urine dilution, chromatographic separation, etc. Therefore, it is not the absolute number of identifications but rather the relative ca. three-times increase of this number that illustrates the merit of the bursting bubble pretreatment. Note that here we deliberately restricted metabolite search to the already known urine metabolites. Some of the observed MS signals not found by the search of urine metabolites could possibly correspond to the yet non-reported urine metabolites. Therefore, the total number of metabolites present in MS might be even higher than the numbers displayed in Fig. 3.

3.3 Characteristics of chemical enrichment by bubble bursting

The detected compounds spanned a broad chemical range (Table S-4; Fig. 4). The majority of compounds was

detected as protonated species ($M+H^+$) with mass-to-charge ratio (*m/z*) between 100 and 600 (Fig. S-1) and displayed R-values from 6 to 12 (Fig. 4). Indeed, R-values are only relevant to the chemicals observed in the both bulk urine and collected aerosol. The absence of sharp variation in R-values between different chemical classes is most likely relevant to the high chemical complexity of urine. It was observed in a previous study that model chemicals displayed notably higher and more diverse R-values when pre-concentrated from single-chemical solutions rather than from a chemical mixture (Chingin et al. 2016). The exact mechanism for the averaging of chemical enrichment in complex solutions remains unknown, but it is evident that inter-molecular interactions play crucial role during chemical partitioning at the bubble surface interface. The smooth profile of chemical enrichment in the bursting bubble approach is important to enrich a broad range of organic compounds without significant chemical discrimination. Chemical discrimination is usually unavoidable during lyophilization, chemical extraction, solute precipitation, ultrafiltration and other pre-concentration approaches.

The highest R-values among the detected urine metabolites were observed for lipids and lipid-like molecules (Fig. 4), and the highest R-values among the lipids and lipid-like molecules were observed for steroids and their derivatives (R = 11–15, Table S-3), which is consistent with the high surface activity of these compounds. Lipids and lipid-like molecules constituted 43 % from the total number of detected metabolites in the collected aerosol. Remarkably, the signals of sodiated species ($M+Na^+$) displayed generally much lower R-values (1–1.5) compared to $M+H^+$ signals (6–12). It was previously observed that the concentration of inorganic salts, such as CsI and $AgNO_3$, in the bursting bubble aerosol could notably decrease relative to the original bulk solution. The degree of inorganics depletion increased with the concentration of organics solutes. It was proposed that the depletion of inorganic solutes could be caused by their partial ejection from the surface interface of rising bubbles by the surface-active organic solutes (Chingin et al. 2016). The decrease of NaCl concentration in bursting bubble aerosol relative to the bulk urine solution can be very roughly estimated as $\langle R_{(M+H)}/R_{(M+Na)} \rangle$ 6–10. The pronounced depletion of NaCl reflects the high concentration of organic solutes in urine, which is necessary for the effect to occur (Chingin et al. 2016).

Overall, these data provide evidence for the notable preconcentration and desalting of organic content in urine by bubble bursting. The effects of organics preconcentration and desalting are equally important to increase the chemical sensitivity of urine analysis. The high concentration of salt in urine is detrimental to
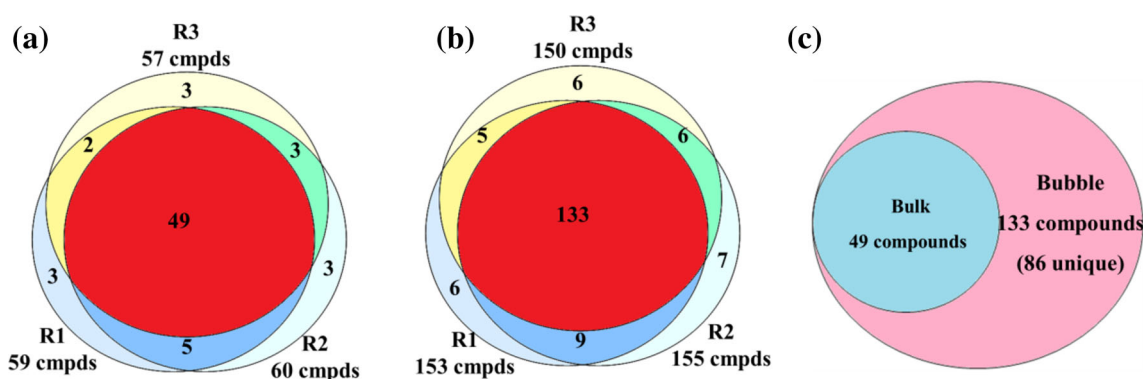
Fig. 3 Venn diagrams presenting the number of metabolites identified in triplicate urine analysis: **a** bulk urine samples; **b** collected aerosol samples produced by bubble bursting of the same bulk samples; **c** comparison between the results of bulk urine analysis and collected aerosol analysis
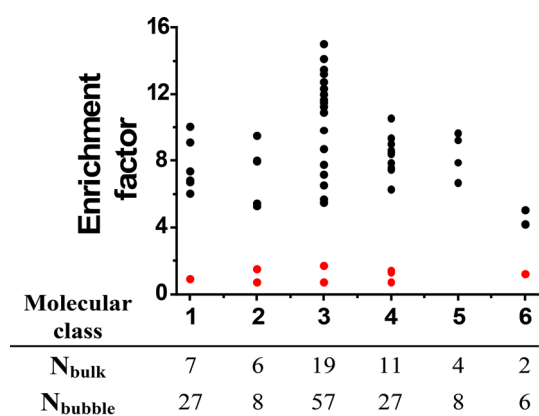

Fig. 4 Intensity enrichment factors for the protonated (*black dots*) and sodiated (*red dots*) MS signals of urine metabolites in the bursting bubble aerosol relative to the original urine sample ($R = I^{bubble}/I^{bulk}$). Metabolites were divided into six groups according to the HMDB "superclass" chemical classification: (*1*) benzenoids; (*2*) organic acids and derivatives; (*3*) lipids and lipid-like molecules; (*4*) organoheterocyclic compounds; (*5*) organooxygen compounds; (*6*) phenylpropanoids and polyketides. The explanation of superclass classification and the entire list of observed metabolites according to this classification are presented in Tables S-3 and S-4

chromatographic columns and MS instruments. Dilution of urine in water (100–1000 times) is usually required prior to analysis. Partial desalting of urine by bubble bursting pretreatment affords lower degree of dilution, thus allowing an additional gain in the sensitivity of analysis.

3.4 Bubble bursting for the higher chemical specificity of analysis

A common metabolomics approach to classify physiological state of a human is based on the statistical analysis of MS data from bodily fluids, e.g. blood or urine. Classification relies on the detection of differential metabolites, i.e. metabolites with statistically significant abundance difference in different physiological states. The higher the number of detected differential metabolites the higher is the specificity and sensitivity of classification.

As the bubble bursting increases the total number of detected metabolites, it also increases the chances of detecting more differential metabolites for more accurate classification. Figure 5a shows the score plot of principal component analysis (PCA) for the MS data (LTQ, Thermo Scientific, San Jose, CA, USA) obtained from urine samples of 24 healthy individuals and from 24 patients with renal insufficiency. Figure 5b shows the score plot for the MS data obtained from the bursting bubble aerosols collected from the same samples and analyzed under the same conditions. The increased degree of data clustering is evident in the score plot of collected aerosols relative to the score plot of bulk urine. The cumulative variance contribution rate for the three major principal components increased from 90 % in bulk to 98 % in aerosol, indicating the increased fidelity of data description by the model. Furthermore, the aerosol samples displayed much higher degree of data separation across the first main component than the bulk samples (Fig. 5a, b). The analysis of loading plots (Fig. 5c, d) showed that for the aerosol samples the first main component was contributed by a larger number of compounds spanning broader molecular mass range than for the bulk samples. No identification of differential metabolites was, however, attempted in this set of experiments due to the lack of hi-res MS data. This example illustrates the utility of bubble bursting approach to enhance the specificity of differentiation for metabolomics data.

3.5 Bubble bursting for the higher sensitivity of molecular detection

The increase in the chemical sensitivity of urine analysis by the bubble bursting pretreatment is also useful for the monitoring of toxic and/or prohibited molecules present in

Fig. 5 Differentiation between healthy people (normal) and patients with renal insufficiency (abnormal) by the PCA of MS data from bulk urine (**a**) and from the aerosols collected by bubble bursting (**b**). Loading plots for three major principal components are shown (**c**, **d**)
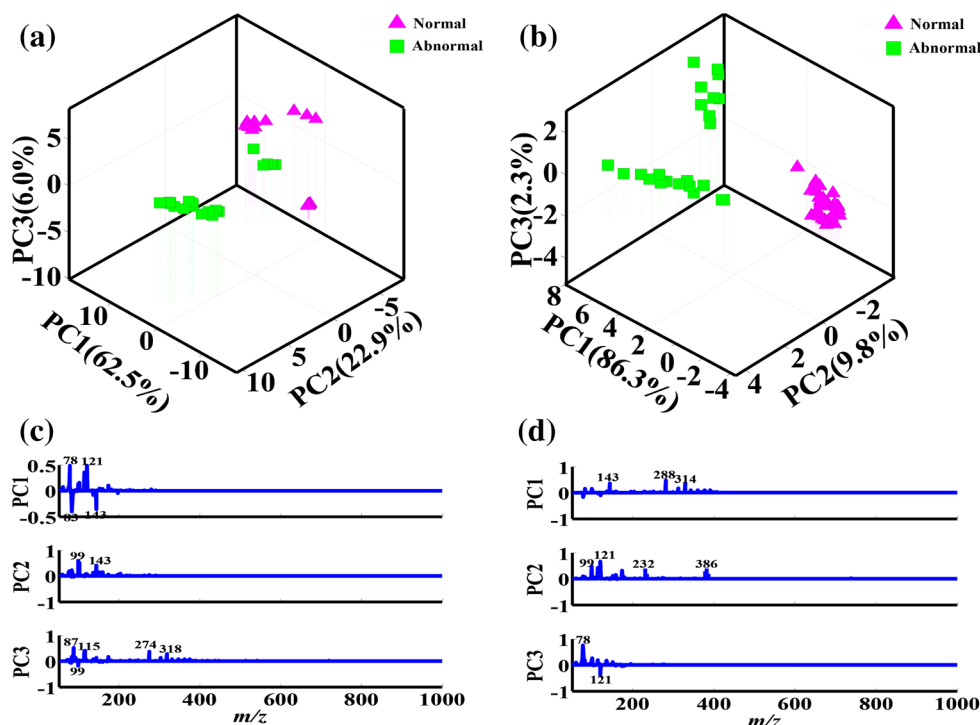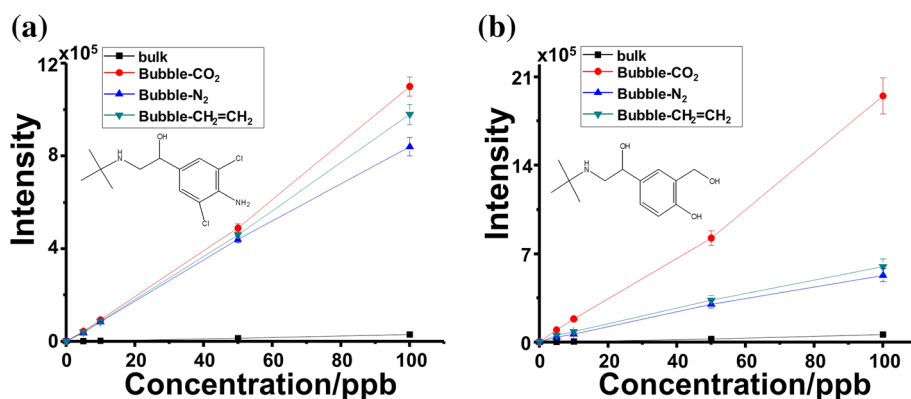
Fig. 6 The enrichment of signal intensity for clenbuterol (**a**) and salbutamol (**b**) in urine by bubble bursting. The results for three different gases (CO_2, N_2, C_2H_4) are compared. Detection was done on LTQ-MS. Drug concentration corresponds to undiluted urine

urine at a trace level. It was previously shown that the lower the analyte concentration the higher its enrichment factor. The decrease of enrichment factor at increasing solute concentration is probably related to the chemical saturation of bubble surface (Chingin et al. 2016). Figure 6 and Table S-5 show the effect of bubble bursting pretreatment on the MS signal intensity of clenbuterol and salbutamol spiked into urine samples at various known concentrations. Ca. ten-times lower limits of detection (LODs) were observed for the both drugs (Table S-5). Indeed, even stronger effect would have been achieved if the desalting effect was taken into account and the accordingly lower degree of urine dilution was used. Interestingly, chemical enrichment of the two tested drugs revealed quite different dependence on the composition of

bubbling gas (Fig. 6). The magnitude of enrichment for clenbuterol was very similar with either N_2, C_2H_4 or CO_2. In contrast, the enrichment of salbutamol was ca. 3 times stronger in CO_2 than in N_2 and C_2H_4. Analogous enrichment behavior was also observed for melamine in a previous study (Chingin et al. 2016). We propose that the higher enrichment of salbutamol and melamine in CO_2 bubbles could possibly be explained by the stabilization of these molecules at the bubble surface interface via chemical interaction with CO_2 gas. For example, a CO_2 molecule could form a hydrogen bond with one of the hydroxyl groups in salbutamol molecule or even be chelated by the two adjacent hydroxyl groups of salbutamol via the formation of two hydrogen bonds, thus reducing the molecular energy state. Similar, CO_2 molecule could also be chelated

by the two adjacent amino groups of melamine. These results suggest that chemical interaction between the organic solutes adsorbed at the bubble surface interface and the gas molecules inside the bubble can enhance the analyte adsorption to the bubble surface and thereby provide higher chemical enrichment and selectivity of preconcentration. Mechanistic study regarding the role of bubble gas on the process of chemical partitioning at the bubble surface interface is currently underway in our laboratory.

4 Concluding remarks

Bubble bursting has been demonstrated as a simple and versatile approach for the simultaneous preconcentration and desalting of organic solutes in urine. Unlike the common pretreatment routines for sample preconcentration and purification, the bubble bursting approach obviates notable chemical losses as far as the organic content of urine is concerned. Also, quite even profile of chemical enrichment is obtained for different classes of organics. Our results thereby indicate that bubble bursting can be directly integrated as a sample pretreatment step into a broad variety of existing workflows for both targeted and untargeted urine analysis. For example, the preconcentration of metabolites in urine by bubble bursting could be useful in personalized medicine, e.g., for the selection of therapeutic agents and their doses, pharmacokinetics of drugs in the treatment of kidney disorders (diabetic nephropathy, chronic renal failure, transplant-associated complications, oncological disorders), as well as for the monitoring of patients under intensive care (Beger et al. 2016). Another application of the approach could be the search for new potential biomarkers for the early diagnosis of renal disorders (Coon et al. 2008), as well as other conditions associated with kidney dysfunction, such as severe pathology of pregnancy (Kononikhin et al. 2016). Following the current demonstration for urine analysis, the application of bubble bursting can be in future extended for the preconcentration and desalting of organic solutes in other complex biological fluids, such as human plasma, saliva, tissue extracts, lysates, etc.

Compliance with ethical standards

Conflict of Interest All the authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the research committee of the East China University of Technology and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

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