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Development of a metabolomic approach based on urine samples and direct infusion mass spectrometry

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ABSTRACT

The analysis of urine by direct infusion mass spectrometry suffers from ion suppression due to its high salt content and inter-sample variability caused by the differences in urine volume between persons. Thus, urine metabolomics requires a careful selection of the sample preparation procedure and a normalization strategy to deal with these problems. Several approaches were tested for metabolomic analysis of urine samples by direct infusion electrospray mass spectrometry (DI–ESI–MS), including solid phase extraction, liquid–liquid extraction, and sample dilution. In addition, normalization of results based on conductivity values and statistical treatment was performed to minimize sample variability. Both urine dilution and solid phase extraction with mixed mode sorbent considerably reduced the salt content in urine, providing comprehensive metabolomic fingerprints. Moreover, statistical data normalization enabled the correction of inter-sample physiological variability, improving the quality of results obtained. Therefore, high-throughput DI–ESI–MS fingerprinting of urine samples can be achieved with simple pretreatment procedures allowing the use of this noninvasive sampling in metabolomics. Finally, the optimized approach was tested in a pilot metabolomic investigation of urine samples from transgenic mice models of Alzheimer's disease (APP/PS1) in order to illustrate the potential of the methodology.

Metabolomics, defined as the comprehensive measurement of all metabolites in a sample, is a powerful tool in systems biology research, connecting the genome with the final phenotype via physiological development and through interactions with the environment [1]. The potential of this approach has been demonstrated in numerous fields, including nutritional studies, disease biomarkers discovery, and drug development [2,3]. Methodologically, the conventional use of nuclear magnetic resonance is being progressively replaced by mass spectrometry because this technique offers several advantages such as higher sensitivity, capability for identification and quantification of metabolites, and suitability for application to complex samples [4]. The coupling of mass spectrometry with separation techniques is frequently reported in order to obtain simpler spectra and facilitate the interpretation of metabolic fingerprints [5], but these hyphenated approaches also present several drawbacks, such as long time of analysis and analytical bias, depending on the selected metabolomic platform [6]. Alternatively, sample introduction by direct infusion into the mass spectrometry system (DIMS)[1] has been recently proposed in disease studies with serum and plasma samples [7–11]. Although this approach presents several drawbacks related to the lack of resolution for isobar differentiation and difficulty of quantification without stable isotope internal standards, the usefulness of this screening tool has been demonstrated in terms of wide metabolome coverage and fast analysis. For these reasons, the application of this high-throughput methodology to urine samples may be of great interest in disease diagnosis due to the easy availability and noninvasive sampling of this biological fluid, which well reflects the health state of organisms [12]. Urine samples are normally collected as 24-h samples in order to provide a complete picture of excretion, but random single-spot urine collection is more convenient in clinical

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[1] *Abbreviations used:* DIMS, direct infusion into the mass spectrometry system; SPE, solid phase extraction; LLE, liquid–liquid extraction; DI–ESI–MS, direct infusion electrospray mass spectrometry; SD, sample dilution; AD, Alzheimer's disease; RP–SPE, reverse phase extraction; NP–SPE, normal phase extraction; MM–SPE, mixed mode extraction; IS, ion spray; DP, declustering potential; FP, focusing potential; PLS–DA, partial least squares discriminant analysis; LOESS, locally weighted scatterplot smoothing; RSD, relative standard deviation.

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practice despite the higher biological variance [13]. The analysis of urine samples by mass spectrometry presents two important problems related to ion suppression caused by this salty matrix [14] and inter-sample variability due to the differences in the volume of urine excreted between different individuals [15]. Therefore, sample preparation is critical in order to minimize ion suppression [16], removing salts from urine samples and minimizing possible perturbations of metabolites to obtain comprehensive fingerprints. Solid phase extraction (SPE) has become one of the most important techniques for the treatment of biological fluids in metabolomics [17], using both reverse and normal phases, and ion exchange matrices [18,19]. However, the development of mixed mode sorbents, which involve multiple retention mechanisms by the incorporation of different ligands into the same sorbent, allows the extraction of a wider fraction of metabolites [20] and represents a promising alternative in sample treatment for metabolomic fingerprinting. In addition, liquid–liquid extraction (LLE) has also been considered for the treatment of this complex matrix for the separation of nonpolar or low-polar analytes despite being a rare extraction technique in metabolomics [13]. On the other hand, normalization of results allows the reduction of biological variability caused by physiological factors such as hydration status, diet, microflora, and diurnal cycle that require the use of a correction parameter that does not systematically vary along the demographic groups of interest [21]. In this sense, measurements of ionic conductivity and osmolality have been proposed as indicators of total endogenous metabolic output, reflecting the dilution rate of urine samples [22]. Another conventional normalization parameter for urine is creatinine concentration because, under normal conditions, urinary creatinine excretion is relatively constant and measurable [23], although it varies depending on age, sex, diet, physical exercise, mental state, and kidney impairment, among other factors [24]. In addition, different statistical methods for data preprocessing can also be performed to normalize results by scaling the spectra to the same virtual overall concentration [25,26].

There are a few metabolomic studies related to the use of DIMS in urine samples, mainly in nutritional [27–29] and toxicological [30] areas, but there is still a need to delve into possible approaches that overcome the critical issues mentioned above.

For this purpose, in this work several tools for sample treatment and data normalization in metabolomic analysis of urine samples were evaluated using direct infusion electrospray mass spectrometry (DI–ESI–MS). Several methods for urine treatment in order to reduce ion suppression were studied using SPE with different sorbents, LLE, and sample dilution (SD). Moreover, ionic conductivity measurements and statistical preprocessing were compared for data normalization. To compare the effectiveness of these approaches for group classification based on DIMS fingerprinting results, urine samples were studied from both male and female volunteers collected at different times of the day (fasting morning and after lunch). Finally, the most suitable approach was tested in a pilot study with urine samples from transgenic mice models of Alzheimer's disease (AD) (APP/PS1) in order to illustrate the potential of the methodology.

Materials and methods

Reagents and materials

All solvents used were high-performance liquid chromatography (HPLC) grade. Methanol and chloroform were purchased from Aldrich (Steinheim, Germany). Acetic acid, ammonium acetate, and dichloromethane were supplied by Merck (Darmstadt, Germany). Ammonia solution (32%) was obtained from VWR (Leuven, Belgium). Water was purified with a Milli-Q gradient system (Millipore, Watford, UK). The SPE cartridges used were 3-ml columns filled with 500 mg of DSC-18 (Supelco) for reverse phase extraction (C18), Bond Elut SI (Varian) for normal phase extraction (silica), and Isolute Multimode (IST) combining nonpolar (C18), strong cation exchange (SO_3^-), and strong anion exchange (NR_3^+) solid phases.

Sample collection

Two different sets of samples were used in this study. Human urine samples, collected from 11 volunteers (5 male and 6 female) fasting and after lunch, were used for evaluating the suitability of the different treatment and normalization procedures tested in this study. The second set was urine samples from transgenic mice models of AD (APP/PS1) and wild-type controls employed to validate the optimized methodology in a pilot study for the search of potential biomarkers of this disorder. APP/PS1 double transgenic mice (C57BL/6 background) were generated by crossing homozygotic PS1M146L transgenic mice with heterozygotic Thy1-APP751SL mice (Swedish [K670N and M671L] and London [V717I] FAD mutations) (Charles River, France) as described previously [31]. On the other hand, age-matched wild-type mice of the same genetic background (C57BL/6) were purchased from Charles River Laboratory for their use as controls (wild type). Male animals at 6 months of age were used for experiments (10 specimens per group). All samples were immediately frozen to −80 °C and stored until analysis.

Sample treatment

Three different strategies were tested for simultaneous desalting and extraction of metabolites from urine samples. In all cases, urine was thawed at room temperature and immediately centrifuged to remove particulate matter (6000 rpm, 10 min, 4 °C). Before extraction, pH of these samples was adjusted to 7.0.

Sample dilution

In SD, urine was diluted 10-fold with a 50% (v/v) methanol/water mixture.

Liquid–liquid extraction

In LLE, 500 µl of urine was mixed with the same volume of organic solvent (chloroform or dichloromethane), followed by vigorous vortexing for 5 min and centrifugation at 6000 rpm for 3 min at room temperature, in order to collect the clean organic phase for analysis. Alternatively, 750 µl of a mixture of urine, methanol, and organic solvent (1:1:1, v/v/v) was vigorously shaken by vortexing

Table 1
Experimental conditions for SPE procedures.

	RP–SPE	NP–SPE	MM–SPE
Conditioning	2 ml methanol	2 ml methanol	2 ml methanol
Equilibration	2 ml water	2 ml methanol 5%	–
Sample loading	1 ml urine	1 ml urine	1.5 ml urine
Washing	2 ml water	2 ml methanol 95%	2 ml water
Elution	1 ml methanol	1 ml methanol 5%	(i) 0.5 ml methanol (ii) 0.5 ml ammonium acetate 10 mM (pH 3.0) (iii) 0.5 ml ammonia 5% (in methanol)

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