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Urinary metabolic fingerprinting of mice with diet-induced metabolic derangements by parallel dual secondary column-dual detection two-dimensional comprehensive gas chromatography☆

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ABSTRACT

This study investigates the potential of a parallel dual secondary column-dual detection two-dimensional comprehensive GC platform (GC × 2GC–MS/FID) for metabolic profiling and fingerprinting of mouse urine. Samples were obtained from a murine model that mimics a typical unhealthy Western diet featuring both high fat and sugar (HFHS) intake, which induces obesity, dyslipidemia, and insulin resistance. Urines collected at different steps of the study were used to obtain pivotal and comparative data on the presence and relative distributions of early markers of metabolic disease. The data elaboration and interpretation work-flow includes an advanced untargeted fingerprinting approach, with peak-region features to locate relevant features to be quantified by external standard calibration. The reliability of untargeted fingerprinting is confirmed by quantitative results on selected relevant features that showed percentages of variations consistent with those observed by comparing raw data quantitative descriptors (2D peak-region volumes and percent of response). Analytes that were up-regulated with % of variation ranging from 30 to 1000, included pyruvic acid, glycerol, fructose, galactose, glucose, lactic acid, mannitol and valine. Down-regulation was evidenced for malonic acid, succinic acid, alanine, glycine, and creatinine. Advanced fingerprinting also is demonstrated for effectively evaluating individual variations during experiments, thus representing a promising tool for personalized intervention studies. In this context, it is interesting to observe that informative features that were not discriminant for the entire population may be relevant for individuals.

1. Introduction

Early changes in metabolite profiles of biofluids (e.g., plasma and urine samples) are considered reliable biomarkers of early metabolic dysfunction and often are used to characterize clinical manifestations of metabolic disorders, mainly type 1 and type 2 diabetes. A key pathogenic mechanism is the disruption of glucose homeostasis, which leads to the development of insulin resistance and impaired insulin production. Disturbances in both the secretion and action of insulin impact the global regulation of metabolism, affecting the composition of blood, urine, and other body fluids. Traditionally, to get a vision of the physiopathologic responses related to metabolic glucose deregulation, single metabolites or classes of small molecules are measured using targeted analytical assays. In that approach, the relationships among diverse metabolites and multiple pathways are ignored, hindering a useful integrated vision in the assessment of complex diseases. More recently, the identification of potential disease biomarkers has been greatly facilitated by the upsurge in new technologies for comprehensive metabolic profiling, which are often collectively termed metabolomics [1–3]. For instance, recent epidemiological studies used metabolomics to predict incident diabetes and revealed branched-chain and aromatic amino acids, including, isoleucine, leucine, valine, tyrosine, and phenylalanine, as highly significant predictors of future diabetes [3,4].

In this context, two-dimensional comprehensive gas chromatography with mass spectrometry (GC × GC–MS) represents one of the most advanced and informative hyphenated GC platforms currently available for medium-to-low molecular weight metabolite profiling. Thanks to its superior separation power, sensitivity,

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and informative bi-dimensional (2D) separation patterns, detailed profiles and fingerprints of complex biological samples can be comprehensively evaluated. However, to reveal the so-called *metabolic fine print* [4], analytical efforts must be directed to low molecular weight organic compounds (<1500 Da) with a great diversity of chemical properties and wide concentration ranges. As a consequence, robust, reproducible, accurate, and informative methods are needed to enable reliable samples comparisons.

From this perspective, when a GC × GC–MS platform is adopted, the system configuration represents a critical but challenging aspect requiring careful tuning of the columns' stationary phase chemistry and dimensions (especially length and inner diameter) to maximize the system separation power and simultaneously avoid second-dimension (^{2}D) column overloading, thereby improving quantitation accuracy and response linearity over a wider range of concentrations [5]. Quantitative metabolomics, which includes not only the detailed profiling of metabolites but also their true quantitation, is required to realize the potential of biomarker investigations.

To date, most studies of metabolic profiling by GC × GC–MS have used a conventional column setup consisting of a non-polar primary (^{1}D) column of 30 m × 0.25 mm d_c × 0.25 μm d_f and a single mid-polarity secondary (^{2}D) column of 1–2 m × 0.1 mm d_c × 0.1 μm d_f [6]. However, to overcome some limits of conventional column configurations in these earlier studies, Koek et al. demonstrated that wider bore ^{2}D columns (i.e., 0.25 mm d_c) with higher mass loadability provided more precise and accurate quantitative results, although the overall system peak capacity was lower [6]. More recently, Rocha et al. [7] investigated the composition of human urine volatilome, adopting an apolar (DB-5) ^{1}D column of 30 m × 0.32 d_c × 0.25 μm d_f coupled to a polar (DB-FFAP) ^{2}D column of 0.79 m × 0.25 d_c × 0.25 μm d_f. That column setup provided appropriate orthogonality and suitable mass loadability for the analytes under study.

Generally, GC × GC detection requires fast detectors, for example flame ionization detector (FID) or electron impact (EI) fast acquisition time-of-flight mass spectrometers (TOFMS). However, reliable and consistent results both in terms of analyte identification and quantitation also can be obtained with modern fast quadrupoles, operating at high frequencies [8,9]. These MS detectors are experiencing a growing popularity in GC × GC applications, confirmed by the increasing number of publications appearing in the literature [10]. Last but not least, high-resolution TOFMS (HR-TOFMS) detectors are emerging as valuable tools in hyphenated multidimensional analytical platforms for metabolomics because of their informative potential in analyte identifications based on accurate mass detection [11].

This study investigates the potential of a parallel dual secondary column-dual detection two-dimensional comprehensive GC platform (GC × 2GC–MS/FID) for metabolic profiling and fingerprinting of urine samples obtained from a murine model of diet-induced metabolic derangements. The animal model mimics a typical unhealthy Western diet featuring both high fat and sugar (HFHS) intake, which induces obesity, dyslipidemia, and insulin resistance [13,14]. Urine samples collected at different times during the study were used to obtain pivotal and comparative data on the presence and relative distribution of early markers of metabolic disease.

The instrumental platform operates at close-to-optimal ^{2}D linear velocities in both chromatographic dimensions and doubles secondary column loading capacity with positive effects on overall system orthogonality, efficiency, and selectivity [12]. In addition, the improved information potential due to the dual detection (by MS and FID), poses challenges in terms of data elaboration but offers the opportunity to cross-validate results of both targeted-quantitative and untargeted profiling.

The data elaboration and interpretation work-flow includes an advanced untargeted fingerprinting approach with peak-region features [11,15,16] to locate relevant features to be quantified by external standard calibration. Accuracies of both targeted and untargeted elaboration are assessed by comparing MS and FID results. The advantages of dual detector/dual pattern information cross-matching in terms of exploiting the overall system potential for comparative analysis and quantitative metabolomics are apparent in the results.

2. Experimental

2.1. Chemicals

All chemicals were from Sigma–Aldrich (Milan, Italy), in particular:

(a) pure standards of *n*-alkanes (from *n*-C9 to *n*-C25) for system evaluation, flow/pressure optimization, and linear retention index (I^T_S) determination;
(b) pure standards for quantitative determinations of pyruvic acid, lactic acid, malonic acid, succinic acid, malic acid, 2-ketoglutaric acid, hippuric acid, L-alanine, L-valine, glycine, L-threonine, L-tyrosine, creatinine, phenylalanine, xylitol, ribitol, glycerol, fructose, galactose, glucose, mannitol, and myo-inositol, and the internal standard (ISTD) gallic acid;
(c) derivatization reagents O-methylhydroxylamine hydrochloride (MOX) and N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA);
(d) HPLC-grade solvents: methanol, pyridine, *n*-hexane, and dichloromethane.

2.2. Samples

Four-week-old male C57BL/6J mice (n = 16) (Harlan-Italy; Udine, Italy) were housed in a controlled environment at 25 ± 2 °C. All the animals were fed with a normal pellet diet for 1 week prior to the experimentation. The animals then were allocated to one of two dietary regimens, either normal (control, n = 8) or a high-fat high-sugar diet (HFHS, n = 8), for 12 weeks. The HFHS diet contained 45% fat, 20% protein, and 35% carbohydrate. Animal care was in compliance with the "Principles of laboratory animal care" (NIH publication 85–23, 1985) and the experimental protocol has been approved by the Turin University Ethics Committee. Urine samples were collected at 1 week (basal) and after 6, 9, and 12 weeks (W6, W9, and W12) then immediately quenched on liquid nitrogen and stored at −80 °C until derivatization/analysis. For urine collection, conscious mice were individually placed in metabolic cages with free access to water for 16 h.

Urine samples were submitted to a standard derivatization protocol [17] consisting of the following steps: 200 μL of urine and a suitable volume of ISTD (gallic acid solution at 10 g/L) were diluted with methanol up to 1000 μL and carefully mixed (Whirlimixer vortex, Fisher Scientific, Loughborough, Leicestershire, UK). Then, 30 μL of MOX were added to 20 μL of that solution and the resulting solution was incubated for 2 h at 60 °C. Next, 30 μL of MSTFA were added and the mixture was incubated at 100 °C for 60 min. The resulting sample solution diluted in *n*-hexane was immediately analyzed in duplicate or stored at −80 °C until analysis.

2.3. GC × 2GC–MS/FID instrument set-up

GC × GC analyses were run with the following system configuration: an HT280T multipurpose sampler (HTA, Brescia, Italy) was integrated with an Agilent 6890 GC unit coupled to an Agilent