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Optimization of a liquid chromatography ion mobility-mass spectrometry method for untargeted metabolomics using experimental design and multivariate data analysis☆

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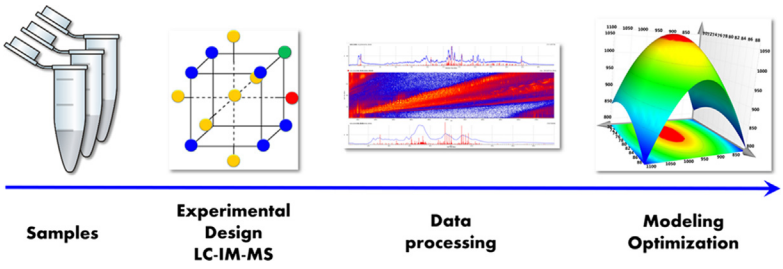
HIGHLIGHTS

- A new optimization approach for LC-IM-MS based metabolomics methods was developed.
- Nine parameters were optimized with an experimental design approach.
- Ionization parameters, chromatographic flow rate, wave velocity and drift gas flow rate were the investigated factors.
- Optimum conditions were defined for both electrospray source and ion mobility settings.

GRAPHICAL ABSTRACT



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ABSTRACT

High-resolution mass spectrometry coupled with pattern recognition techniques is an established tool to perform comprehensive metabolite profiling of biological datasets. This paves the way for new, powerful and innovative diagnostic approaches in the post-genomic era and molecular medicine. However, interpreting untargeted metabolomic data requires robust, reproducible and reliable analytical methods to translate results into biologically relevant and actionable knowledge. The analyses of biological samples were developed based on ultra-high performance liquid chromatography (UHPLC) coupled to ion mobility - mass spectrometry (IM-MS). A strategy for optimizing the analytical conditions for untargeted UHPLC-IM-MS methods is proposed using an experimental design approach. Optimization experiments were conducted through a screening process designed to identify the factors that have significant effects on the selected responses (total number of peaks and number of reliable peaks). For this purpose, full and fractional factorial designs were used while partial least squares regression was

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used for experimental design modeling and optimization of parameter values. The total number of peaks yielded the best predictive model and is used for optimization of parameters setting.

1. Introduction

The concept of "metabolome" refers to the comprehensive analysis of all metabolites present in a given biological system, fluid, cell or tissue [1,2]. Metabolites can be defined as small organic molecules involved in or resulting from enzymatic reactions. So, metabolomics is one of the "omics" approaches based on biochemical and molecular characterizations of the metabolome and the changes in metabolites related to genetics, environment, drugs or diet and other factors [3]. Two different analytical approaches may be used in metabolomics studies: targeted and untargeted. The targeted approach relies on the measurements of a specific subset of metabolites, focusing typically on pathways of interest. However, the untargeted approach has the advantage of simultaneously measuring as many metabolites as possible in a biological sample. To achieve this goal, different analytical strategies have been developed. Most of them are based on nuclear magnetic resonance (NMR) or mass spectrometry (MS) [4]. However, due to the superior sensitivity of mass spectrometry [5], the predominant analytical methods are nowadays based on hyphenated approaches combining chromatographic separation and MS. In metabolomics, the separation step prior to MS analysis reduces the high biological sample complexity and gives access to the differentiation of isomeric species that cannot be easily done by MS alone. This also decreases ion suppression effects and enhances sensitivity. Liquid and gas chromatography are the most commonly used separation techniques [6]. Recently, approaches using gas phase separation, namely ion mobility spectrometry (IMS) [7] are gaining in interest [8–14]. Indeed, integrated with high resolution mass spectrometry (HRMS) and liquid chromatography (LC-IM-MS), IMS provides additional analyte selectivity without significantly compromising the speed of MS-based measurements. The MS dimension affords accurate mass information while the IMS dimension provides molecular, structural and conformational information. Indeed, combining ion mobility spectrometry with hybrid mass spectrometry instruments offers an additional separation dimension for more comprehensive analysis of complex mixtures [10,15–17]. In addition, the drift time determined from the IMS analysis can be converted to the ion collision cross section (CCS) which is an intrinsic property of the analyzed ion and is therefore a very robust parameter that can be used together with the *m/z* determination for compound identification. Furthermore, having access to retention time, mass and molecular density obtained by the combination of LC-IM-MS allows integration of measurements that enhances molecular identification [18]. UHPLC-IM-MS heat map showing the multidimensionality of the data acquisition is presented in Fig. 1. So far, HRMS coupled with pattern recognition techniques is an established tool to obtain comprehensive metabolite profiling from biological datasets [19–21]. However, interpreting untargeted metabolomics data requires robust, reproducible and reliable analytical methods to translate results into biologically relevant and actionable knowledge [22]. This paves the way for new, powerful and innovative diagnostic approaches in the post-genomic era [23–25]. Metabolomics studies include sequential and integrated steps spanning from the biological question to data interpretation. It includes sample storage, sample pretreatment, data acquisition and processing, multivariate statistical modeling, validation, and interpretation. The final result is highly dependent on the quality of each step [3]. Metabolomics being primarily a data-driven and hypothesis generating tool, optimized and standardized protocols for most of these steps are essential to retrieve reliable, reproducible and interpretable information from generated data [26]. Previously described optimization protocols of MS based untargeted metabolomic methods are mainly based on the assessment of endogenous metabolites or signals of added standards [27–30]. However, these optimization approaches are, to some extent, restrictive and dependent on the standards used or endogenous metabolites chosen. To keep the intrinsic and vital property of untargeted metabolomics - which is to cover as much as possible metabolites present in the studied sample - a global optimization approach is needed to take into account all the detectable metabolites. Here we propose a strategy for optimizing untargeted UHPLC-IM-MS methods using an experimental design approach to address this issue. Design of experiments (DoE) is a formalized procedure in which specific and controlled modifications are made to a given system of input variables in order to create predictive mathematical models that allow the optimization of the monitored response variables of the system as a function of the applied modifications. DoE may thus be used to explain the system's changes. The main advantage of using DoE is its ability to generate optimized experimental parameters with a minimum of experiments [31–33]. Hence, DoE is an effective and economical solution to experimental modeling and optimization. A general workflow of an experimental design approach is shown in Fig. 2. The aim of the present study was to optimize the analytical conditions of an LC-IM-MS system using a DoE approach.

2. Experimental

2.1. Reagents and chemicals

Methanol and acetonitrile were purchased from VWR Chemicals (France), ultrapure water (18 MΩ) from Millipore (Molsheim, France) and formic acid from Fluka (Saint Quentin Fallavier, France). The chemicals used were of analytical grade. Leucine Enkephalin (Sigma–Aldrich) at a concentration of 2 ng/L (in acetonitrile/water, 50/50) was used as reference for mass measurements.

2.2. Sample preparation

Four different human sera from volunteers in our laboratory staff were used to create a pooled sample. A small volume of each of the individual samples was mixed into a pooled sample. Two dilutions (1/2 and 1/4) were made from this pool and 100 μL of the pooled sample and dilutions were treated with 300 μL of methanol. The resulting samples were then mixed using a vortex mixer for 20 s, left on ice at 4 °C for 60 min to allow protein precipitation, then centrifuged for 15 min at 15,000× *g*. Supernatant of each sample was dried. Dried extracts were suspended with 100 μL of Acetonitrile/Water 50/50.