Journal of Chromatography A, xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Unravelling the effects of multiple experimental factors in metabolomics, analysis of human neural cells with hydrophilic interaction liquid chromatography hyphenated to high resolution mass spectrometry☆

Víctor González-Ruiz[a,b], Julian Pezzatti[a], Adrien Roux[b,c], Luc Stoppini[b,c], Julien Boccard[a,b], Serge Rudaz[a,b,*]

[a] School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, Geneva, Switzerland
[b] Swiss Centre for Applied Human Toxicology, Switzerland
[c] Tissue Engineering Lab, HEPIA – HES-SO University of Applied Sciences and Arts Western Switzerland, Campus Biotech, Geneva, Switzerland

ARTICLE INFO

Article history:
Received 3 May 2017
Received in revised form 16 October 2017
Accepted 23 October 2017
Available online xxx

Keywords:
Metabolomics
Multifactorial experiments
HILIC-HRMS
Neuroinflammation
Chemometrics
AMOPLS

ABSTRACT

This work introduces a strategy for decomposing variable contributions within the data obtained from structured metabolomic studies. This approach was applied in the context of an *in vitro* human neural model to investigate biochemical changes related to neuroinflammation. Neural cells were exposed to the neuroinflammatory toxicant trimethyltin at different doses and exposure times. In the frame of an untargeted approach, cell contents were analysed using HILIC hyphenated with HRMS. Detected features were annotated at level 1 by comparison against a library of standards, and the 126 identified metabolites were analysed using a recently proposed chemometric tool dedicated to multifactorial Omics datasets, namely, ANOVA multiblock OPLS (AMOPLS). First, the total observed variability was decomposed to highlight the contribution of each effect related to the experimental factors. Both the dose of trimethyltin and the exposure time were found to have a statistically significant impact on the observed metabolic alterations. Cells that were exposed for a longer time exhibited a more mature and differentiated metabolome, whereas the dose of trimethyltin was linked to altered lipid pathways, which are known to participate in neurodegeneration. Then, these specific metabolic patterns were further characterised by analysing the individual variable contributions to each effect. AMOPLS was highlighted as a useful tool for analysing complex metabolomic data. The proposed strategy allowed the separation, quantitation and characterisation of the specific contribution of the different factors and the relative importance of every metabolite to each effect with respect to the total observed variability of the system.

1. Introduction

Metabolomics is now established as an essential tool in the study of complex biological systems, allowing the surveillance of the complex molecular networks that exist at the end of the natural flow of information from genes through mRNA and proteins up to living organisms [1]. Because the time scale at which metabolite levels vary is much faster than that of protein expression, metabolic profiles not only represent the idiosyncrasy of the organism, but they also allow the rapid exploration of the environmental adaptation of living systems [2]. Two major strategies are commonly applied in metabolomics: the targeted and untargeted approaches [3]. On the one hand, targeted workflows are useful to gain insights into hypothesis-driven studies, when the identity of the metabolites expected to change has already been established and such molecules can be selectively monitored. The sample preparation and separation steps can be optimised to maximise the recovery of the target analytes, minimise the interference and improve their separation. Due to their selectivity and sensitivity, QqQ mass spectrometers with various acquisition modes (*e.g.,* SWATH, DDA) are usually employed for such studies. On the other hand, the untargeted approach could be more informative when there is no *a priori*

☆ Selected paper from the 12ème Congrès Francophone sur les Sciences Séparatives et les Couplages Analytiques (SEP 2017), 29–30 March 2017, Paris, France.

* Corresponding author at: School of Pharmaceutical Sciences, University of Lausanne, University of Geneva, University Medical Centre, 1 Rue Michel-Servet, 1205 Geneva, Switzerland.
E-mail address: serge.rudaz@unige.ch (S. Rudaz).

https://doi.org/10.1016/j.chroma.2017.10.055
0021-9673/

knowledge about the changes that occur in the metabolome under study. In this case, generic sample preparation and separation steps are preferable because they allow the recovery of a broader variety of analytes present in the samples. High-resolution mass spectrometers (HRMS) such as orbitrap- and QTOF-based analysers are desirable to maximise detection coverage.

The combination of state-of-the-art analytical techniques and data processing strategies is indeed evolving to make feasible the use of metabolomic approaches to better understand human diseases and improve their prevention and treatment [4,5]. Among others, neuroinflammation processes are well known to participate in neurodegenerative conditions such as Parkinson's and Alzheimer's diseases [6–8]. Although many original alternatives are appearing in this field [9,10], the diagnosis and study of these disorders still require access to samples that are difficult to obtain from patients, except in *post-mortem* examinations [11–13]. Accordingly, there is a need to develop adequate *in vitro* biological models for such pathologies and appropriate methodologies to extract relevant information. In this context, many substances have been reported to exert neurotoxic effects by triggering an inflammatory response in neuronal tissues. One such neurotoxicant is trimethyltin (TMT), which is widely used both in the production of plastic materials and as a biocide and whose neurological effects have been studied extensively [14]. Because TMT can be used to artificially induce an inflammatory response in cell cultures, the use of human neural cultures to conduct metabolomic studies on neuroinflammation has emerged as an appealing way to explore neurodegeneration mechanisms.

Although reverse-phase LC constitutes one of the gold standards in metabolomics, the use of orthogonal separation mechanisms with different selectivities is currently broadening the achievable coverage of small molecules. Among them, HILIC fits remarkably well with the separation performance required by polar compounds such as neurotransmitters and similar small polar metabolites [15–18]. In the recent years, several comparative studies [19–21] and numerous application examples have proven HILIC to be not only a useful standalone technique [22–24], but also as a complementary technique to RPLC to increase the comprehensiveness of metabolomics coverage [25–29]. It has been recently proposed that, when operated under carefully chosen conditions, HILIC can be useful even to separate compounds typically related to RP separations, such as omega fatty acids [30]. However, to the best of our knowledge, this alternative separation method has not yet been fully exploited to conduct neuroinflammation research.

As previously described [31], the design of metabolomic studies typically involves analysing a limited number of samples and simultaneously measuring a large number of analytes. Moreover, the concurrent investigation of several experimental factors constitutes a promising strategy to reliably assess the impact of multiple influences occurring at the same time on a given biological system. The combination of these aspects makes extracting biologically meaningful information from the resulting massive and complex datasets a challenging endeavour. A variety of dimensionality reduction strategies can be employed for this purpose [31], and both unsupervised and supervised analyses of very large data tables in the field of metabolomics have been discussed elsewhere [32]. Multivariate methods based on the computation of latent variables or components, such as principal component analysis (PCA) and partial least squares (PLS) regression constitute potent tools to provide compact data representations and diagnostic tools for the detection of relevant variables and patterns amidst complex data structures [33]. Nevertheless, most of these approaches lack the ability to fully exploit the underlying data structure inherent to the design of a multifactorial study because the influence of different experimental factors is mixed. To overcome this drawback, ANOVA multiblock OPLS (AMOPLS) was recently proposed as a new data analysis tool [34]. This strategy involves an ANOVA-based partitioning of the sources of variation (experimental variables) followed by the joint analysis of ANOVA submatrices by means of a supervised multiblock algorithm based on the OPLS framework and was reported to be a potent approach for analysing multifactorial Omics experiments.

2. Materials and methods

2.1. LC–MS conditions

Chromatography was performed on a Waters H-Class Acquity UPLC system composed of a quaternary pump, a column manager and a FTN auto sampler (Waters Corporation, Milford, MA, USA). The samples were separated on a Waters Acquity BEH Amide column (150 × 2.1 mm, 1.7 μm) bearing an adequate VanGuard pre-column. Solvent A was H_2O:MeCN (5:95, v/v) and solvent B was H_2O:MeCN (70:30, v/v) containing 10 mM ammonium formate (pH = 6.5 in the aqueous part). The pH value of the aqueous component of mobile phase B was carefully monitored to ensure retention time repeatability, and it remained stable for at least 3 days. The following gradient was applied: 0% B for 2 min, increased to 70% B over 18 min, held for 3 min, and then returned to 0% B to re-equilibrate the column for 7 min (total run time was 31 min). The flow rate was 500 μL min^{-1}, and the column temperature was kept at 40 °C. A sample volume of 5 μL was injected. LC–MS sequences were set up as follows: 2 × blank (mobile phase A), 1 × extraction solution, 1 × amino acids standard mix (Sigma-Aldrich), 6 × QC, 36 × samples (randomised, plus 1 QC every 6 samples), 6 × QC, 1 × amino acids standard mix, 1 × extraction solution, 2 × blank.

The UPLC system was coupled to a maXis 3G Q-TOF high-resolution MS from Bruker (Bruker Daltonik GmbH, Bremen, Germany) through an electrospray interface (ESI). The instrument was operated in TOF mode (no fragmentation). The capillary voltage was set at 4.7 kV in positive ionisation mode, the nebulising gas pressure was 2.0 bar, and the drying gas temperature and flow rate were 225 °C and 8.0 L min^{-1}, respectively. The transfer time and pre-pulse storage time were set at 60 and 5.0 μs, respectively. Data between 50 and 1000 m/z were acquired in profile mode at a rate of 2 Hz. ESI and MS parameters were optimised using a mix of representative standards fed by a syringe pump and mixed with the LC eluent (mid-gradient conditions) within a tee-junction. Formate adducts in the 90–1247 m/z range were employed for in-run automatic calibration using the implemented quadratic plus high-precision calibration algorithm (Bruker Daltonik). MS and UPLC control and data acquisition were performed through the HyStar v3.2 SR2 software (Bruker Daltonik) running the Waters Acquity UPLC v.1.5 plug-in.

2.2. Solvents and additives

UPLC–MS-grade methanol, acetonitrile and water were obtained from Fisher (Fisher Scientific, Loughborough, UK). Ammonium formate and ammonium hydroxide for MS were supplied by Sigma-Aldrich (Buchs, Switzerland). UPLC–MS-grade formic acid was obtained from Biosolve (Valkenswaard, The Netherlands).

2.3. Analysis of standards and creation of reference database

Overall, 358 metabolite standards were selected either from the Sigma MSMLS library or among other compounds available in our laboratory (Sigma-Aldrich). Stock solutions of these standards (prepared according to the provided instructions in the case of the MSMLS library, otherwise as 1 mg mL^{-1} methanolic solutions) were mixed as required, evaporated in a rotatory concentrator

Please cite this article in press as: V. González-Ruiz, et al., Unravelling the effects of multiple experimental factors in metabolomics, analysis of human neural cells with hydrophilic interaction liquid chromatography hyphenated to high resolution mass spectrometry, J. Chromatogr. A (2017), https://doi.org/10.1016/j.chroma.2017.10.055