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Metabolic fingerprinting of high-fat plasma samples processed by centrifugation- and filtration-based protein precipitation delineates significant differences in metabolite information coverage

Thaer Barri [a,*], Jens Holmer-Jensen [b], Kjeld Hermansen [b], Lars O. Dragsted [a]

[a] Department of Human Nutrition, Faculty of Life Sciences, University of Copenhagen, Rolighedsvej 30, DK-1958 Frederiksberg, Denmark
[b] Department of Endocrinology and Metabolism MEA, Aarhus University Hospital, Tage-Hansens gade 2, DK-8000 Aarhus C, Denmark

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ABSTRACT

Metabolomics and metabolic fingerprinting are being extensively employed for improved understanding of biological changes induced by endogenous or exogenous factors. Blood serum or plasma samples are often employed for metabolomics studies. Plasma protein precipitation (PPP) is currently performed in most laboratories before LC–MS analysis. However, the impact of fat content in plasma samples on metabolite coverage has not previously been investigated. Here, we have studied whether PPP procedures influence coverage of plasma metabolites from high-fat plasma samples. An optimized UPLC-QTOF/MS metabolic fingerprinting approach and multivariate modeling (PCA and OPLS-DA) were utilized for finding characteristic metabolite changes induced by two PPP procedures; centrifugation and filtration. We used 12-h fasting samples and postprandial samples collected at 2 h after a standardized high-fat protein-rich meal in obese non-diabetic subjects recruited in a dietary intervention. The two PPP procedures as well as external and internal standards (ISs) were used to track errors in response normalization and quantification. Remarkably and sometimes uniquely, the fPPP, but not the cPPP approach, recovered not only high molecular weight (HMW) lipophilic metabolites, but also small molecular weight (SMW) relatively polar metabolites. Characteristic SMW markers of postprandial samples were aromatic and branched-chain amino acids that were elevated ($p < 0.001$) as a consequence of the protein challenge. In contrast, some HMW lipophilic species, e.g. acylcarnitines, were moderately lower ($p < 0.001$) in postprandial samples. LysoPCs were largely unaffected. In conclusion, the fPPP procedure is recommended for processing high-fat plasma samples in metabolomics studies. While method improvements presented here were clear, use of several ISs revealed substantial challenges to untargeted metabolomics due to large and variable matrix effects.

1. Introduction

In order to evaluate the functionality of MS-based metabolomics platforms for profiling and fingerprinting of biological samples, an artificial mixture of a wide spectrum of standard metabolites is often used [1,2]. This mixture simulates real complex samples usually encountered in biochemistry where the constituents vary in concentration, polarity, pKa, and chemical properties. Such standard metabolite mixture in addition to a pooled biological sample can be employed for monitoring overall platform performance and data quality. Metabolite response normalization by a structurally analogous stable isotopically labeled internal standard (SIL-IS) [3] or a structurally similar internal standard (IS) [4] would provide more quantitative information. However, it is not possible to add useful internal standards for all compounds in a complex mixture, such as blood plasma, and differential matrix effects would also influence metabolite normalization [5] as well as QTOF/MS accuracy in the mass measurements [6].

ESI-based MS detection and quantification is often confronted with endogenous and exogenous matrix effects, which might suppress or enhance metabolite ionization signals [7]. Therefore, (semi-) quantification with accurate mass measurements in untargeted metabolomics by using QTOF/MS is difficult in case no actions are taken to overcome or correct for the matrix effects. Matrix effects can be reduced by powerful chromatographic separation such as ultra-high pressure separations and 2D-LC [8], by proper calibration [9] using SIL-IS, standard addition calibration, or both, or by sample cleanup to remove matrix components [5].

The QTOF/MS technique is known for its powerful capability of performing qualitative measurements with high resolution

* Corresponding author. Tel.: +45 35332372; fax: +45 35333245.
E-mail address: thbar@life.ku.dk (T. Barri).

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and mass accuracy. The technique has also proven capabilities in quantification of diverse chemical moieties in biological samples [10–13].

Rigorous sample clean-up is not generally preferred for untargeted metabolite profiling, but it is applied to limit profiling to specific classes of compounds as in lipidomics [14]. One clean-up procedure is almost inevitably used for LC-QTOF/MS profiling of plasma or serum samples, namely removal of proteins to avoid damage to the analytical column and MS capillaries. Serum/plasma protein precipitation (PPP) in "omics" research has been traditionally carried out by using simple solvent precipitation and then centrifugation [15–17]. High-fat plasma samples will have different physical–chemical properties than normal plasma samples. Accordingly, the procedure used for PPP of high-fat plasma samples may influence the metabolite profile.

We have used an external metabolite standard mixture of 44 components and a mixture of seven internal standards for two purposes. First, we have evaluated the performance of two UPLC-QTOF/MS methods prior to metabolomic fingerprinting of high-fat human plasma samples after two different protein precipitation procedures, a centrifugation-based (cPPP) and a filtration-based (fPPP). Second, we have investigated the possibility of normalizing metabolite responses to SIL-IS or IS in spiked pooled study samples and in study samples for potential (semi-)quantification in untargeted metabolomics. The overall aim was to implement a fully characterized platform for applications in nutritional metabolomics. The specific objective of the present study was to apply metabolomics fingerprinting accompanied with multivariate analysis (PCA and OPLS-DA) to establish a reliable LC–MS separation method and PPP procedure accompanied with use of metabolite standards, and to apply the developed platform to pattern recognition and biomarker discovery in a set of plasma samples collected under fasting and postprandial conditions, the latter after a high-fat load.

2. Experimental

2.1. Materials and methods

Acetonitrile and methanol (Optima grade) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Acetone and formic acid were ordered from Sigma–Aldrich (Sleeze, Germany). 96-Well Sirrocco plates (Sirocco™ plates, #186002448) for protein precipitation and filtration (a 0.45-μm nylon-based filter incorporated) and 96-well collection plates (#186002481) for extract collection and subsequent sample analysis were bought from Waters Corporation (Hedenhusene, Denmark). The chromatographic columns BEH C18 (100 mm × 2.1 mm, 1.7 μm particle size) and HSS T3 (C18, 100 mm × 2.1 mm, 1.8 μm particle size) were purchased from Waters. Reagent water was ion-exchanged and purified further by a Millipore (Billerica, MA, USA) unit to obtain an electrical resistance below 18 MΩ.

2.2. Metabolite external and internal standard mixtures

A standard mixture of 44 biologically relevant metabolites and another mixture of 7 ISs have been prepared (see Figs. 1 and 2 and Text_SI file). The standard mixture was developed for quality control of the analytical platform, such as monitoring changes in mass accuracy, retention time, and instrumental sensitivity drifts. The standard mixture was also implemented for investigating its potential use for metabolite response normalization to some structurally related SIL-ISs for semi-quantification in untargeted metabolomics analysis. The internal standards were chosen to cover the retention time and polarity range of the standard metabolite mixture as well as the plasma samples.

Table 1
Mobile phase gradient parameters used in the two chromatographic methods.

Chromatography Method	Time (min)	Flow (mL min^{-1})	%B	Gradient curve	Column temperature
(I) BEH C_{18}	0.0	0.4	0.0	Initial	65 °C
	5.0	0.4	100	6	
	5.5	0.4	0.0	6	
	6.0	0.4	0.0	6	
(II) HSS T3 C_{18}	0.0	0.5	5	Initial	50 °C
	1.0	0.5	8	11	
	2.0	0.6	15	10	
	3.0	0.7	40	10	
	4.0	0.8	70	6	
	4.5	1.0	100	6	
	5.0	1.2	100	1	
	6.4	1.2	100	1	
	6.6	1.0	5.0	1	
	6.8	0.5	5.0	6	
	7.0	0.5	5.0	1	

2.3. UPLC-QTOF/MS

2.3.1. UPLC

An ultra-performance liquid chromatography (UPLC) system coupled to orthogonal acceleration quadruple time-of-flight (Premier QTOF) mass spectrometer (Waters Corporation, Manchester, UK) was used for sample analyses. *Chromatography Method I*: The analytical UPLC column was BEH C_{18}. The mobile phase consisted of aqueous (A) and organic (B) solvent components, where A was 0.1% formic acid in reagent water and B was 0.1% formic acid in a mixture of 80% acetonitrile and 20% acetone (run time 6 min). *Chromatography Method II*: The UPLC column used was HSS T3 C_{18}. The mobile phase was 0.1% formic acid in reagent water (A) and 0.1% formic acid in 70% acetonitrile and 30% methanol (B). For this method, a gradient of both mobile phase solvent and flow rate was used (run time 7 min). Mobile phase gradient and other conditions related to the two methods are summarized in Table 1. The injection mode for both methods was partial loop with needle overfill and the injected sample volume was 5 μL with an injection loop size of 10 μL.

2.3.2. QTOF/MS

Electrospray ionization (ESI) in negative and positive modes was employed for the analysis. The selected mass range was from 50 to 1000 m/z in full scan mode with a scan time of 0.08 s and an inter-scan delay of 0.02 s. Ion source and desolvation gas (nitrogen) temperatures were 120 and 350 °C (*Chromatography Method I*) and 120 and 400 °C (*Chromatography Method II*), respectively. The sampling cone and desolvation gas flow rates were 30 and 800 L h^{-1} (*Chromatography Method I*) and 50 and 1000 L h^{-1} (*Chromatography Method II*), respectively. Capillary probe voltage was set at 3.2 and 2.8 kV for positive and negative mode analysis, respectively. Sampling cone voltage was set at 30 kV. Leucine enkephalin (500 ng mL^{-1} and flow rate of 0.05 mL min^{-1} split before entering the ion source) was infused intermittently every 10 s and utilized as a lock mass for accurate on-line mass calibration. To get more structural information, a low-to-high collision energy ramp (MS^E mode) was implemented for selected samples. The MS^E collision energy was ramped between 10 and 35 V during each individual scan of 0.08 s with an inter-scan delay of 0.02 s.