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Influence of sample preparation on lipidomics analysis of polar lipids in adipose tissue

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ABSTRACT

The main limitations of lipidomics analysis are the chemical complexity of the lipids, the range of concentrations at which they exist, and the variety of samples usually analyzed. These limitations particularly affect the characterization of polar lipids owing to the interference of neutral lipids, essentially acylglycerides, which are at high concentration and suppress ionization of low concentrated lipids in mass spectrometry detection. The influence of sample preparation on lipidomics analysis of polar lipids in adipose tissue by LC–MS/MS was the aim of this research. Two common extractants used for lipids isolation, methanol:chloroform (MeOH:$CHCl_3$) and methyl *tert*-butyl ether (MTBE), were qualitatively and quantitatively compared for the extraction of the main families of lipids. The obtained results showed that each family of lipids is influenced differently by the extractant used. However, as a general trend, the use of MTBE as extractant led to higher extraction efficiency for unsaturated fatty acids, glycerophospholipids and ceramides, while MeOH:$CHCl_3$ favored the isolation of saturated fatty acids and plasmalogens. The implementation of a solid-phase extraction (SPE) step for selective isolation of glycerophospholipids prior to LC–MS/MS analysis was assayed to evaluate its influence on lipids detection coverage as compared to direct analysis. This step was critical to enhance the detection coverage of glycerophospholipids by removal of ionization suppression effects caused by acylglycerides.

1. Introduction

Lipids constitute a wide variety of biological molecules involved in essential functions due to their role as structural components of cell membranes, energy storage, and intermediates in signaling pathways [1–4]. For this reason, lipidomics, a branch of metabolomics devoted to the qualitative/quantitative analysis of the lipidome, has experienced a drastic expansion [3]. Because of their biological importance, lipids are under tight homeostatic control and exhibit spatial and dynamic complexity at multiple levels [5,6]. Thus, it is not surprising that altered lipid metabolism plays a key role in the pathogenesis of common diseases.

Adipose tissue is a complex, essential, and highly active metabolic and endocrine organ [2,7,8] whose lipid composition can be classified into two main groups: the neutral or non-polar lipids and the polar lipids. Neutral lipids, composed by acylglycerides (tri-, di- and monoacylglycerides), cholesteryl esters and cholesterol, constitute the most abundant group, while polar lipids with specific functional groups encompass essentially free fatty acids, the different families of glycerophospholipids and ceramides [8]. Adiposity, which represents the fraction of total body mass composed by neutral lipids, is closely related with key physiological parameters such as blood pressure, systemic insulin sensitivity, and concentration of molecules such as serum

Abbreviations: ACN, acetonitrile; BPC, base peak chromatogram; CEs, ceramides; $CHCl_3$, chloroform; CID, collision-induced dissociation; DGs, diacylglycerides; DDA, data-dependent acquisition; D-PBS, Dubelcco's Phosphate Buffered Saline; FAs, fatty acids; HMDB, Human Metabolome Database; IPA, 2-isopropanol; MFs, molecular features; MGs, monoacylglycerides; MTBE, methyl *tert*-butyl ether; PAs, phosphatidic acids; PCs, glycerophosphatidylcholines; PEs, glycerophosphatidylethanolamines; PGs, phosphatidylglycerol; Pis, glycerophosphatidylinositols; PLs, plasmalogens; PS, phosphatidylserines; SLs, sphingolipids; SMs, sphingomielyns; TGs, triacylglycerides

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triglycerides and leptin [2,9,10]. Thereby, the excess of adipose tissue or obesity, particularly in the visceral compartment, is associated with insulin resistance, hyperglycemia, dyslipidemia, hypertension, and prothrombotic and proinflammatory states [2,7,11]. On the other hand, polar lipids are the main components of biological membranes, also in adipose tissue, and regulate cellular signaling to facilitate the transmission of biological information across them [12,13]. Additionally, polar lipids act as bioactive mediators that have been recognized as endogenous regulators of key cellular processes. Most of these bioactive mediators originate from the cleavage of lipid constituents of cellular membranes under the activity of phospholipases. Adipose tissue function, which has a crucial role in the development of obesity-related comorbidities including insulin resistance and non-alcoholic fatty liver disease, is dysregulated in obese individuals with a key function of polar lipids [14]. According to these associations, it is important to study the composition of adipose tissue under different biological circumstances or conditions.

Main lipid species in human serum, liver and adipose tissue have already been characterized in several studies [15]. For this purpose, LC–MS/MS has proved a noticeable detection capability for analysis of the different families of lipids [8,9,16–20], except for free fatty acids that are better characterized by GC–MS after derivatization [21–24]. Nevertheless, LC–MS/MS is able to offer quantitative response for the most concentrated fatty acids found in biological samples. Concerning sample preparation this critical step determines the type and concentration of isolated lipids and, for this reason, several preparation methods have been applied to biological samples with the goal of improving overall lipid coverage. The methods have been based on sample preparation techniques such liquid–liquid extraction, precipitation using organic solvents, and solid-phase extraction (SPE). Folch et al. [25] and Bligh–Dyer [26] proposed methods for general extraction of lipids based on methanol (MeOH) and chloroform ($CHCl_3$) mixtures as extractants. These two methods differ in the extractants ratio —concretely (1:2, *v/v*) or (2:1, *v/v*) MeOH:$CHCl_3$ for the Folch and Bligh–Dyer methods, respectively—, and the extractant–sample ratio. Specifically, the Folch method employs a higher extractant–sample ratio (roughly a 20-fold excess of extractant) as compared to the Bligh–Dyer protocol. According to the literature, the use of a MeOH:$CHCl_3$ mixture for lipids extraction ensures the isolation of all major lipid classes, which are mostly enriched in the chloroform phase [27,28]. Later, Matyash et al. introduced a novel extraction procedure for isolation of lipids using as extractant a MeOH and methyl *tert*-butyl ether (MTBE) mixture in a 1.5:5 (*v/v*) ratio, then adding water to improve the separation step. This methodology avoids the use of a toxic and carcinogenic extractant as chloroform, thus reducing the environmental burden as well as the health risks for the exposed personnel [29]. This protocol, tested in four biological matrices, provided similar to or even better recoveries than either the Folch or Bligh–Dyer methods for major lipid classes [29]. In any case, these overall extraction protocols are characterized by a dual limitation that affect in a different way to the two main lipid families in adipose tissue. Thus, neutral lipids are not quantitatively extracted with these protocols in complex samples such as adipose tissue, thus limitating the identification of neutral lipids to the most concentrated species. On the other hand, the detection of polar lipids, less concentrated, is critically affected by the presence of neutral lipids that considerably exert ionization suppression effects. For this reason, Baker et al. proposed a dual extraction step for independent extraction of neutral lipids and polar lipids [8]. The first family was extracted by an isooctane: ethyl acetate solution using a protocol based on the work from Hutching et al., which allows separating approximately 90% of neutral lipids from polar lipids [30]. The latter were isolated by a modified Bligh-Dyer method with further clean-up of co-extracted neutral lipids by SPE with silica gel as sorbent [30]. A limitation of this protocol is associated to the concentration of acetic acid used to favor the isolation of polar lipids. This concentration could promote degradation of certain families of minor polar lipids such as plasmalogens [8].

Considering the limitations in the analysis of the different families of lipids in adipose tissue, which specially affect polar lipids, this research was planned to evaluate the influence of sample preparation on the determination of polar lipids in visceral adipose tissue. Two different extractants, MeOH:$CHCl_3$ and MTBE, were tested to compare their efficiency for the extraction of polar lipids but also their inefficiency for extraction of acylglycerides: the main interferents in the detection of polar lipids. Additionally, the implementation of an SPE step with a selective sorbent for retention of glycerophospholipids was assessed to check its influence on the subsequent detection of this family of lipids.

2. Materials and methods

2.1. Chemicals and reagents

Chromatographic mobile phase B was prepared using LC − MS grade acetonitrile (ACN) and 2-propanol (IPA) from Sigma–Aldrich (Madrid, Spain). MS-grade formic acid from Scharlab (Barcelona, Spain) and ammonium acetate from Sigma–Aldrich were used as ionization agents for LC–MS/MS analysis. Deionized water (18 MΩ·cm) supplied by a Milli-Q water purification system from Millipore (Bedford, MA, USA) was used to prepare the chromatographic aqueous phase (phase A). Chromatographic grade $CHCl_3$, MeOH, and MTBE from Scharlab were used for sample preparation. The buffer employed in this research for tissue homogenization was Dulbecco's phosphate buffered saline (D-PBS) from Lonza (Basilea, Switzerland).

For identification of free fatty acids (FAs) the injection of commercial standards of these compounds in MeOH was necessary owing to the scant MS/MS information generated by fragmentation. The following commercial standards of FAs were acquired from Fluka Analytical (Buches, Switzerland): lauric (C12:0), myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1), hepatadecanoic (C17:0), stearic (C18:0), oleic (C18:1), linoleic acid (C18:2), linolenic (C18:3), eicosanoic (C20:0), eicosadienoic (C20:2n6), eicosatrienoic (C20:3n6), eicosatetraenoic (C20:4), arachidonic (C20:4), behenic (C22:0), docosatetraenoic (C22:4), docosapentanoic (C22:5), docosahexaenoic (C22:6) and tetracosenoic (C24:0) acids.

2.2. Adipose tissue samples

100 mg of visceral adipose tissue were obtained from a pool of individuals with morbid obesity undergoing bariatric surgery and recruited by the Lipids and Atherosclerosis Unit of the Reina Sofia University Hospital (Cordoba, Spain). The subjects who participated in this study gave written informed consent. The study was conducted according to the Declaration of Helsinki [31] and was approved by the Reina Sofia Hospital Biomedical Research and University of Cordoba Biomedical Experimentation Ethical Committees.

2.3. Apparatus and instruments

A Teflon homogenizer (Kimble Kontes Vineland, NJ, USA) was used for homogenization of adipose tissue samples. A Visiprep™ SPE vacuum manifold (Supelco, PA, USA) with disposable liners (Supelco, PA, USA) was used for enrichment of glycerophospholipids isolated from adipose tissue. A Concentrator Plus speed-vac from Eppendorf (Hamburg, Germany) was used to evaporate the MeOH phase after SPE elution to concentrate the sample, and a vortex shaker from IKA (Wilmington, NC, USA) was used for sample agitation.

An Agilent 1200 Series LC system coupled to an Agilent 6540 UHD Accurate-Mass QTOF hybrid mass spectrometer (Santa Clara, CA, USA) was used for analysis of the extracts. The QTOF detector was equipped with a dual electrospray ionization source for simultaneous spraying of chromatographic eluate and a reference solution to calibrate