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Evaluation of extraction and normalisation strategies for the analysis of lipids in placental vessels

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ABSTRACT

The analysis of lipids in tough or fibrous biological tissues can be challenging due to difficulties in obtaining a representative sample following homogenisation of the tissue. Furthermore, the choice of normalisation method can have a major effect on the quality of quantitative results. Therefore, a range of mechanical homogenisation techniques and normalisation strategies were evaluated for application to human placental vessels. The findings showed that rotor-stator homogenisation in a suitable solvent and wet weight normalisation were the best combination of procedures for quantitative analysis of lipids in placental blood vessels.

1. Introduction

Endogenous bioactive lipids are involved in numerous physiological processes of the human organism and are therefore increasingly subject to quantitative studies in a variety of biofluids and tissues. While the preparation of liquid samples (for example blood plasma) for analysis is relatively simple, extraction of lipids from tissue samples often poses a challenge, particularly when the tissue is made up of fibrous, muscular or similar tough biological material. A prerequisite for a successful analysis is the appropriate choice of a homogenisation and normalisation strategy. An efficient homogenisation method should lead to disruption of the sample resulting in an increased surface area to facilitate quantitative extraction of analytes [1,2].

Placental vessels, especially chorionic plate vessels, have a high muscular and fibrous content resulting in increased toughness [3] and our initial work with this tissue revealed that it was difficult to obtain homogeneous disruption prior to extraction. Typically, lipid contents are normalised to wet tissue weight but we also noted that a range of different normalisation strategies had been applied to fibrous tissues, including dry weight, protein content, total lipid content, or cross-sectional area. Normalisation to protein content has been recommended by some authors in preference to dry or wet weight, as this factor is less susceptible to changes between sample groups and stable structural proteins account for most proteins in tissue samples [4,5]. Furthermore, marker proteins for a specific tissue type have also been used, e.g. creatine or myofibrillar protein content for skeletal muscle samples [6]. It has been demonstrated that the measured concentration of analytes can change considerably depending on the selected normalisation method [5–7]. Tissue wet weight includes intra- and extracellular water but might also be distorted by rinsing of blood-stained tissue or by residual blood, especially in highly perfused tissues such as placental blood vessels. The variability of the water content can be eliminated by freeze-drying the sample pellets after the extraction of lipids.

Hence, we evaluated four homogenisation methods (Dounce homogenisation, ball mill, cryogenic grinding, rotor-stator homogenisation) and three normalisation methods (wet tissue weight, dry tissue weight, protein content) for the extraction and quantitative analysis of oxylipins from human placental vessels.

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2. Material and methods

2.1. Tissue collection

Placentae were collected after obtaining informed consent from healthy pregnant women delivering at full term gestation. Ethics approval was granted by Derby Research Ethics Committee (REC Reference No. 09/H0401/90). First branch chorionic plate arteries (CPA) were dissected, briefly rinsed with PBS, stripped of remaining water droplets using forceps and snap-frozen for storage at −80 °C within 1 h after delivery.

2.2. Comparison of tissue homogenisation methods

CPA from four placentae were minced on dry ice, mixed to form one homogeneous batch of tissue and then transferred into Eppendorf tubes in 0.3 g aliquots. The tissue was subsequently homogenised using one of the four following methods: For ball milling (n = 6), 2 grinding balls (stainless steel, 4 mm ∅) were added to each of the Eppendorf tubes. The tissue was disrupted (MM301 mixer mill, Retsch, Haan, Germany) by milling 7 times for 5 min at 30 s^{-1} with intermittent cooling, until no further visible disruption of the tissue was noted. For the Dounce homogenisation (n = 6), a thawing tissue aliquot was homogenised in a cooled Dounce homogeniser for 2 min. For the cryogenic grinding using liquid N_2 (n = 5), frozen tissue was packed into several layers of cooled aluminium foil and crushed using a pestle and mortar. For the rotor-stator homogenisation (n = 5), frozen tissue was transferred into a glass tube containing 1 mL of ice cold H_2O. The tissue was homogenised for 10 s using a rotor-stator homogeniser (T-25 Ultra-Turrax™, IKA, Staufen, Germany).

After disruption of the tissue using the described methods, ice cold extraction solvent (0.3% formic acid, 0.07% *v/v* butylhydroxytoluene in ethanol) was added to each sample and the tissue was extracted for 1 h at 4 °C on a shaker. The final solvent composition for samples of all methods before extraction on the shaker was 75% extraction solvent and 25% water. Samples were centrifuged at 15,000*g* for 20 min at 4 °C and the supernatant was added to chilled H_2O. The final solvent composition for supernatants of samples of all methods before solid phase extraction (SPE) was 25% extraction solvent and 75% water.

Tissue homogenate supernatants were extracted using SPE columns (Phenomenex Strata-XL 100 μm, polymeric sorbent) according to the instructions of the manufacturer. All samples were disrupted and extracted on the same day and snap frozen before analysis. A previously validated LC–MS/MS method was used for the relative quantification of 10 hydroxyeicosatetraenoic acids (HETEs), 4 epoxyeicosatrienoic acids (EETs), 4 dihydroxyeicosatrienoic acids (DHETs), 2 hydroperoxyeicosatetraenoic acids (HpETEs), 2 octadecadienoic acids (ODEs), 2 leukotrienes, and 6 prostaglandin derivatives [8]. The peak areas were determined and normalised against the initial tissue wet weight. LC–MS/MS-analysis of the samples occurred within one single run.

2.3. Comparison of normalisation methods

CPA from three placentae were minced on dry ice, mixed to form one homogeneous batch of tissue and 12 tissue aliquots were weighed in a range of 300 ± 150 mg. CPA tissues were homogenised using a rotor-stator homogeniser, processed using solid phase extraction (SPE) and analysed via LC–MS as described above. Tissue pellets were kept at −80 °C for determination of protein content and dry weight. Pellets were left to thaw on ice and were briefly disrupted using a pipette tip. 1% SDS was added to each tube and pellets were vortexed for 30 min at 20 °C with 14,000 rpm (vortex at room temperature was necessary to prevent precipitation of sample components). Samples were centrifuged for 20 min at 20 °C with 15,000*g*. The supernatant was diluted 1:10 in 0.9% NaCl and the protein content was determined using a BCA assay. After protein resolubilisation, tissue pellets were freeze-dried using a Thermo Powerdry PL3000 freeze dryer and the dry weight was recorded. The peak areas were determined and normalised against the initial tissue wet weight, dry weight, or protein content.

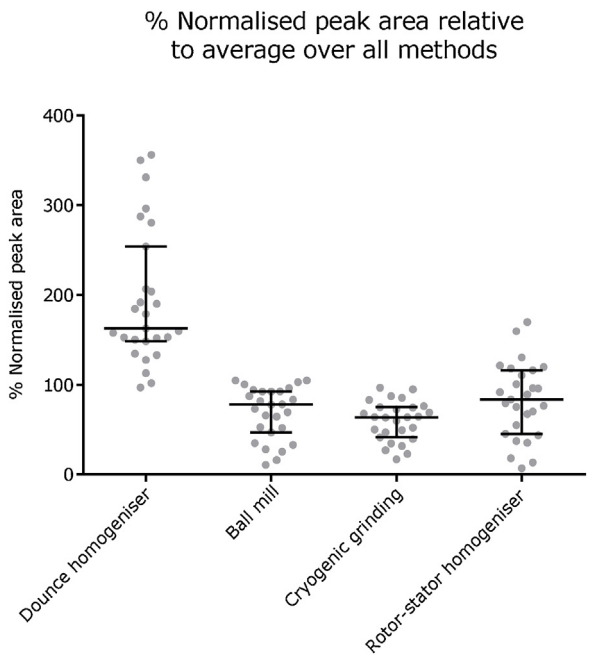

Fig. 1. Median and interquartile range of normalised peak areas of 27 analytes. Each dot represents one analyte, showing the mean of the technical replicates. The mean normalised peak area of each analyte of all technical replicates over a specific method was expressed as percent to the mean normalised peak area of all samples over all methods. Normalisation of the peak area was performed against tissue wet weight (g).

2.4. Data analysis

LC–MS/MS data analysis was done as described in Ref. [8]. Raw data was processed using Analyst (version 1.4, Applied Biosystems, Foster City, USA). Statistical analysis was performed using SPSS software (Version 22, IBM, New York, USA). Graphs were created using Prism (version 6, GraphPad, La Jolla, USA).

3. Results and discussion

3.1. Comparison of tissue homogenisation methods

27 oxylipin analytes were detected in the CPA tissue samples and their relative yields using the different homogenisation methods are shown in Fig. 1.

The Dounce homogeniser did not visibly disrupt the tissue and produced a highly variable recovery of the lipids. Cryogenic grinding and use of the ball mill resulted in the same problem of a hardly disrupted sample, as assessed by visual inspection, so that the subsequent extraction was essentially performed on unhomogenised tissue samples. Both methods were executed while constantly cooling the tissue using liquid N_2. Instead of the anticipated brittle consistency, the tissue samples became tough and rubbery. The rotor-stator homogenisation was the only method realising a disruption of the tissue into fine particles. In essence, the degree of disintegration of the tissue did not seem to affect the extraction efficiency. Therefore, consideration needs to be given to the fact