Lipidome of extracellular vesicles in hypoxic pancreatic cancer

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INTRODUCTION

Pancreatic cancer (PC) is among the malignancies with the poorest prognosis. It is typically asymptomatic in its early stages, making prompt diagnosis challenging using standard clinical screening methods. **Pancreatic ductal adenocarcinoma (PDAC)** accounts for approximately 90% of PCs cases and is predominantly diagnosed at an advanced stage, resulting in a five-year survival rate as low as 7% [1]. Several types of blood tests are considered for PDAC screening [2], such as **carbohydrate antigen (CA) 19-9**, either alone or combined with other blood proteins. However, **magnetic resonance imaging**, **computed tomography**, **endoscopic ultrasound**, and **positron emission tomography** remain the most reliable tools for PC diagnosis in clinical practice. **Circulating tumour DNA** and **extracellular vesicles (EVs)** are emerging as promising tools for the early diagnosis of PDAC. This study focuses on the **lipidomic profiling** of EVs, particularly focusing on **exosomes**—lipid bilayer vesicles secreted by cells into the extracellular space. EVs are classified as **exosomes (30–150 nm)**, **microvesicles (50–1000 nm)**, and **apoptotic bodies (≥500 nm)** [3]. By analysing the lipid composition and concentration in exosomes, we aim to identify novel circulating biomarkers for early PDAC detection. Such biomarkers could offer a significant improvement over current diagnostic strategies, potentially enhancing prognostic accuracy and therapeutic outcomes.

EXPERIMENTAL

As a preliminary step, we compared two lipid extraction protocols to optimize the method for all subsequent sample analyses: the conventional **Bligh and Dyer (B&D) (Figure 1A)** protocol and an alternative one based on a **methanol:methyl tert-butyl ether:water (MeOH:MTBE:H_2O, 4:5:5, v/v/v)** solvent system. After extraction, the organic phase was evaporated under a nitrogen stream, then reconstituted for analysis. Samples were analyzed using **hydrophilic interaction liquid chromatography (HILIC)** coupled with **negative electrospray ionization and linear ion trap mass spectrometry (HILIC-ESI/LIT-MS)**. HILIC enables the separation of lipids based on the polarity of their head groups, allowing compounds from the same lipid class to co-elute. With the aim of maximizing **lipid class coverage for downstream lipidomic profiling**, we compared the extraction efficiency of the two methods. Our results demonstrated that the B&D protocol yielded a broader spectrum of lipid classes, enabling a more comprehensive identification of the lipid species present in the sample. The resulting chromatographic profile is shown in **Figure 1 plot A**. The major phospholipid classes identified were **phosphatidylglycerol (PG)**, **phosphatidylinositol (PI)**, **phosphatidylethanolamine (PE)**, and **phosphatidylcholine (PC)**. These compounds eluted in a consistent order, reflecting their increasing polarity, with **PG eluting first**, followed by **PI**, **PE**, and **PC**. This order of elution is typical for phospholipids under HILIC conditions, where separation is primarily driven by the hydrophilicity of the head group.

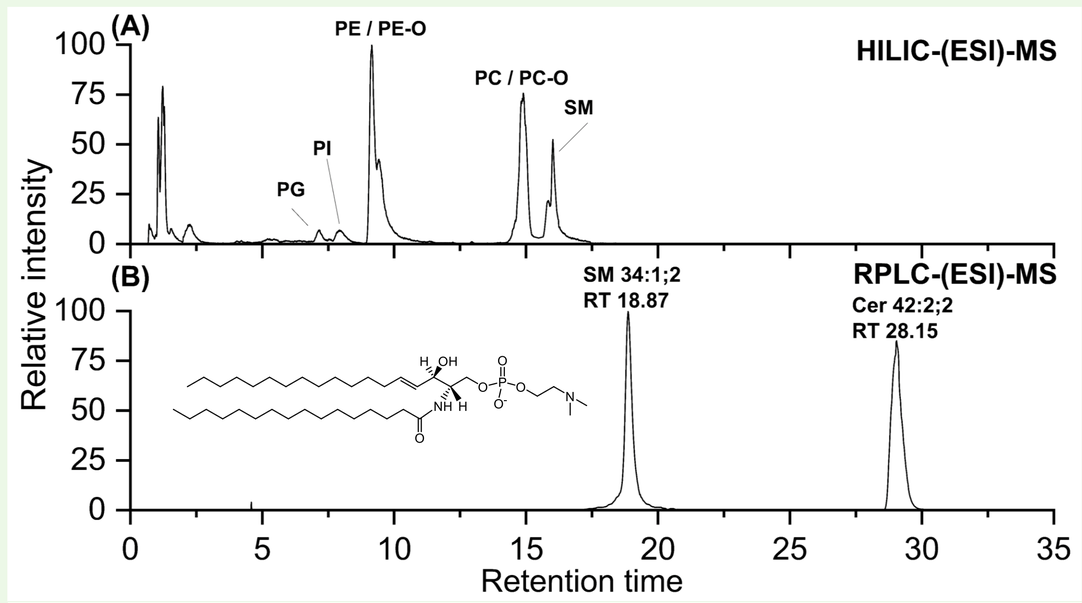


Figure 1: (A) HILIC-ESI(-)-ITMS total ion current chromatogram acquired for the B&D lipid extract of EVs (B) RPLC-ESI(-)-MS analysis of hydrolyzed extract

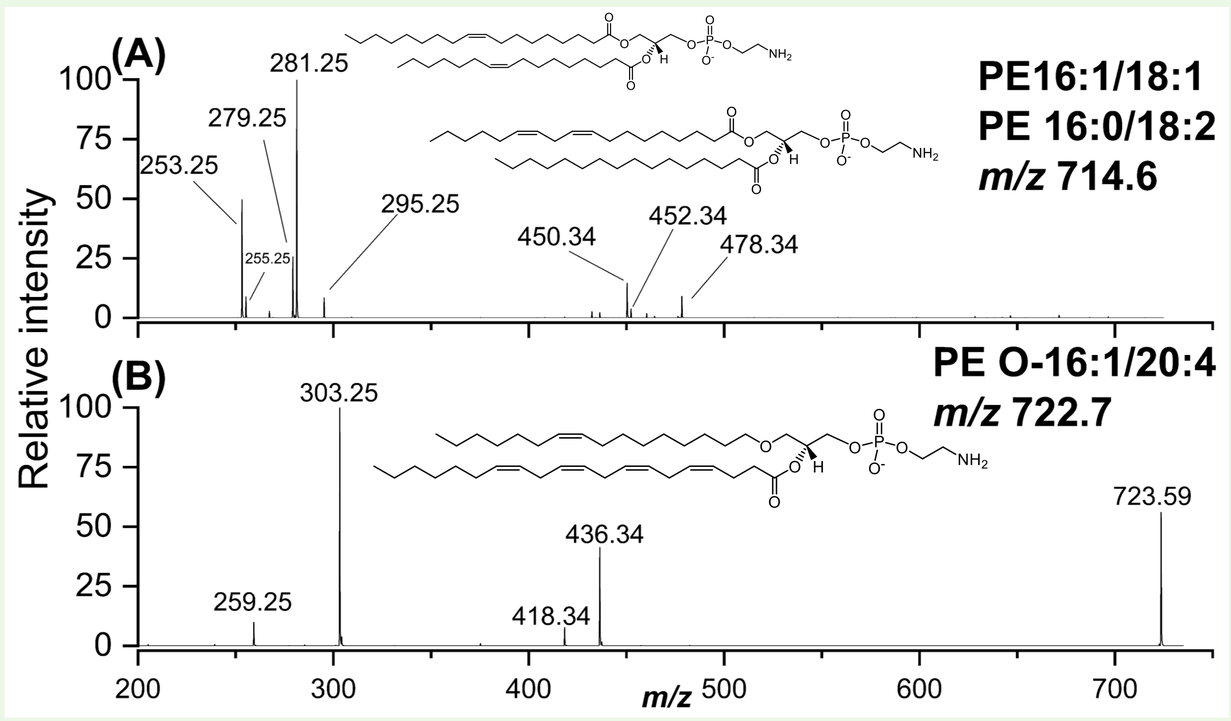


Figure 2. MS/MS spectrum of $[M-H]^-$ ion of PE 34:2 (A) and PE O-36:5 (B). MS/MS data were exploited to determine the position of fatty acyl/alkyl residues on the glycerol backbone. Notably, two major co-eluting isomers were recognized for PE 34:2

FA	16:1	16:0	18:2	18:1	18:0	20:4	20:3	20:2	20:1	22:6	22:5	22:4	24:6	24:5
m/z	253.3	255.3	279.3	281.3	283.3	303.3	305.3	307.3	309.3	327.3	329.3	331.3	355.3	357.3
PE	1.4	2.3	2.4	29.0	7.6	20.6	4.6	2.3	4.2	11.6	5.7	6.6	0.8	0.9
PC	7.6	47.3	3.9	33.0	0.0	2.4	1.3	1.0	2.1	0.6	0.4	0.5	0.0	0.0

Table 1: Estimated fatty acid (FA) distribution in PC and PE classes using the pseudo-DIA approach. Red boxes indicates lower abundant fatty acids, green boxes indicates higher abundant fatty acids.

RESULTS

In order to fully characterize the lipid species detected, **tandem mass spectrometry (MS/MS)** was employed to collect fragmentation spectra of selected precursor ions. This approach allowed us to investigate **diagnostic fragmentation patterns**, providing insight into the structural composition of lipids, including the **position and identity of fatty acyl chains**, and their **regiochemistry** [5]. Such structural information is particularly valuable in differentiating isobaric or isomeric lipid species and in identifying **functionally distinct subclasses**, such as **plasmalogens** and **ether-linked phospholipids**. In the first part of this study, we focused on the **regiochemical identification** of key phospholipid classes, including **PEs (Figure 2 plot A)** and their plasmalogen forms **PE-O (Figure 2 plot B)**, **PCs**, and **PIs**. Interestingly, MS/MS analysis revealed that the samples were particularly **abundant in PE-Os**, which are known to play important roles in membrane structure and cellular signalling providing biological insights in the context of the broader study.

An additional strategy to investigate these samples involved comparing the **fatty acid profiles** across different lipid classes. To this end, we implemented a simplified **pseudo-DIA method** to rapidly gain compositional understandings. For each lipid class, we applied an **isolation window of 100 Da**, centred on the average *m/z* of that class (e.g., *m/z* 750 for phosphatidylethanolamines, PEs). The resulting **MS/MS spectra** were acquired in the ***m/z* range of 200–400**, allowing us to detect fragments corresponding to FAs. It is important to note that the **intensity of these ions is influenced by the positional** on the glycerol backbone. As such, this approach does **not yield quantitative results**, but it is particularly useful for **comparative purposes** across samples.

As an illustrative example, we analysed and compared the **fatty acid composition** of **PE and PC species**, as shown in *Table 1* [4]. The resulting profiles revealed clear differences in **unsaturation levels** and **chain length distribution** between the two classes.

To characterize the **sphingolipid content** of the samples, we developed a specific workflow involving **alkaline hydrolysis** followed by **reversed-phase liquid chromatography (RPLC) (Figure 1B)**. The hydrolysis step selectively removes glycerophospholipids, thereby enriching sphingolipids and improving their chromatographic resolution. The **extracted ion chromatogram (EIC)** shown in Figure 1B illustrates the signal corresponding to selected sphingolipid species, highlighting their retention and relative abundance across the analysed samples. This method proved effective and reproducible, enabling targeted analysis of sphingolipids within the overall lipidomic profiling strategy.

CONCLUSION

We developed and optimized a comprehensive workflow for the lipidomic analysis of extracellular vesicles, suitable for comparative studies across different biological conditions. Among the tested protocols, the **Bligh and Dyer extraction protocol** was selected for its broad lipid class coverage and reproducibility. For **glycerophospholipids**, HILIC-MS combined with targeted MS/MS and **pseudo-data-independent acquisition (pseudo-DIA)** enabled the rapid assessment of fatty acid composition, providing useful comparative information across samples. To specifically address **sphingolipid analysis**, we implemented an additional approach involving **alkaline hydrolysis** followed by **RPLC-MS**, which effectively enriched sphingolipids and enabled their selective detection. Together, these integrated methods provide a solid platform for the characterization of major lipid classes and their comparison between healthy and diseased conditions. This workflow will now be applied to all samples within the broader project to support biologically meaningful interpretations.

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