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# Separation and identification of phospholipids by hydrophilic interaction liquid chromatography coupled to tandem high resolution mass spectrometry with focus on isomeric phosphatidylglycerol and bis(monoacylglycero)phosphate

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ABSTRACT

Changes in lipid composition of cells or tissue are often linked to various diseases. Studies indicate alterations of bis(monoacylglycero)phosphate (BMP) species in diseases such as cancer. Therefore, an extended phospholipid profiling method based on hydrophilic interaction liquid chromatography (HILIC) coupled to high-resolution mass spectrometry (MS) and data-dependent MS/MS acquisition was developed to separate and unambiguously identify BMP species. Lipid species identification was based on retention time, accurate mass and specific MS/MS fragments. The developed method was applied in a proof of concept study to lipid extracts of a cell culture model of conditional oncogene overexpression in MCF-7/NeuT breast cancer cells. Comparison of control and oncogene-induced MCF-7/NeuT breast cancer cells showed changes in BMP species distribution. Thereby, a shift from long-chain to shorter-chain fatty acid composition in BMP species was detected.



## 1. Introduction

Lipids comprise a diverse group of biomolecules with various functions in many different organisms. Nevertheless, the role of lipids is less investigated compared to other 'omics'-fields such as proteomics or metabolomics. In recent years, the importance of lipids for the understanding of numerous diseases and pathological conditions has been recognized, which is why lipidomics has gained in importance and thus, various analytical techniques and methods have been developed [1].

Phospholipids (PL) are one of the most important cellular lipid category. They consist of a central glycerol backbone, which is bound to a phosphate group in *sn*-3- and one to two fatty acid (FA) moieties in *sn*-1- and *sn*-2-position [2,3]. The classification into PL classes is based on their different head group, which is linked to the phosphate group. Additionally, diversity within each PL class is generated by combination of different FA with variation of chain lengths and double bond numbers. PL play a variety of crucial biological functions in many organisms. They are the main structural components of cell membranes and of the respiratory chain in mitochondria [4]. Moreover, they are important for the functionality of membranes or membrane proteins such as ion channels [5] and serve as precursor molecules for second messengers [6]. In addition, the roles of PL alterations in various diseases like cancer were described [6,7].

Among the different PL classes, the constitutional isomer of phosphatidylglycerol (PG), bis(monoacylglycero)phosphate (BMP) (Fig. 1), was erroneously detected as PG until its discovery by Body and Gray in 1967 [8]. BMP is a unique PL for several reasons [9]. First, the active configuration of BMP esterifies FA at the unusual *sn*-2 and *sn*-2′ positions of glycerol backbone compared to all other PL 's at *sn*-1 and *sn*-2 positions [9–14]. Furthermore, FA composition is predominantly characterized by oleic acid (18:1n-9, OA) and docosahexaenoic acid (22:6n-3, DHA) in most cells [15–19], which is why the FA composition seems to be crucial for its biophysical properties as well as for its biochemical function [9,20]. The share of BMP is only 1% of all PL in most mammals and cells [9], however, higher concentrations can be found in specific cell types like lung alveolar macrophages with up to 18% of all PL [9,21]. Furthermore, within cells BMP is highly enriched in membranes of the endosomal/lysosomal compartment [9,22–26]. Related to

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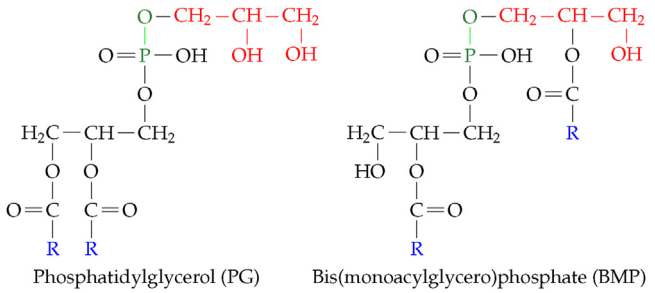


**Fig. 1.** Structural differences of constitutional isomeric PG (left) and BMP (right). Fatty acids are linked to *sn*-1 and *sn*-2 position of PG glycerol backbone, whereas in BMP one fatty acid is bonded at glycerol backbone as well as glycerol head group in *sn*-2 and *sn*-2′ position. R are not further defined fatty acids.

the specific localization, BMP is involved in various functions in organisms and it is critical for the correct function of the endosomal/lysosomal compartment, where biomolecules like lipids or proteins are degraded to smaller, recyclable units [25,24,9]. Also, BMP is involved in dynamic membrane processes like invagination or back-fusion of internal vesicles [9,11,12,14,20,27]. Additionally, BMP is responsible for cellular cholesterol transport and distribution [24].

BMP plays a significant role in further diseases, such as cancer [6,28–30], lysosomal storage diseases, atherogenesis or anti-PL syndrome [9], as well as neuronal ceroid lipofuscinosis [14]. The role of BMP in the pathogenesis in these diseases is not clear or not fully understood. However, studies showed specific alterations in BMP concentration or BMP species in cells or tissues, thus pointing out the possibility to use BMPs as a biomarker for at least some diseases [24,31].

Further development of mass spectrometry (MS) combined with electrospray ionization (ESI) revolutionized the analysis of lipids and is today one of the most important techniques for identification and quantification of lipid species. Additionally, tandem MS of lipid species provides structural information of fatty acid composition in negative as well as head group information in positive ionization mode [32]. Two approaches with different advantages and drawbacks can be used for lipidomics: direct infusion MS (shotgun MS) or liquid chromatography-MS. Due to their identical molecular mass and their similar fragment spectra in negative ionization mode, a distinction of the constitutional isomers PG and BMP solely with MS is not possible. Therefore, prior liquid chromatographic separation of these lipid classes is mandatory. Reversed phase (RP) offers the opportunity to separate lipid species based on their hydrophobic moiety such as fatty acids. In contrast, normal phase and hydrophilic interaction liquid chromatography (HILIC) achieve separation of lipid classes according to their polar head groups. This enables the advantage for quantification due to similar retention times of analytes and internal standards. Quantification in lipidomics is still challenging, because there is a lack of stable isotope labeled standards for each lipid species due to the high complexity of lipidomic samples. In addition, the utilization of selected exogenous lipids with an unusual fatty acid composition is also possible. Furthermore, HILIC avoids ion suppression effects between lipid classes and the assignment of lipid species to a specific class is straightforward [33,34]. Therefore, both RP [35,36] and HILIC [37,38] methods have been applied for separation of BMP and PG. In recent years, supercritical fluid chromatography (SFC) has been increasingly used for lipid separation [34]. The advantages of SFC are based on higher diffusion coefficients and lower viscosities compared to LC. Therefore, SFC provides alternative separations of nonpolar and polar lipid classes in short analysis times [39–42]. Similar stationary phases as in HPLC have been used for lipid analysis in SFC. For example, a separation and quantification method of 30 lipid classes from 6 lipid categories within 6 minutes was recently developed using a silica HILIC column [41].

In this paper, a method development for separation and structural annotation of PL and especially of constitutional isomers BMP and PG by means of HILIC-ESI-MS/MS is described. Subsequently, the applicability of the PL profiling method is demonstrated by analysis of a cell culture model of oncogene-induced senescence in MCF-7/NeuT breast cancer cells in a proof of concept study. This model was chosen because previous work had shown an altered PL pattern in the senescence state and accumulation of cholesterol in the endolysosomal compartment [6], where BMP is reported to exert key functions [9,24,25].

## 2. Materials and methods

### 2.1. Chemicals and materials

1,2-Dipalmitoyl-*sn*-glycero-3-phosphate (PA 16:0/16:0), 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphate (*lyso*-PA 16:0), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (PC 16:0/16:0), 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (*lyso*-PC 16:0), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (PE 16:0/16:0), 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1′-*myo*-inositol) (PI 16:0/16:0), 1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-serine (PS 16:0/16:0), and 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (PG 16:0/16:0) were purchased from Biomol GmbH (Hamburg, Germany). 1,2-Dioleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (PG 18:1/18:1) and cardiolipin (CL) extract of bovine heart were obtained from Sigma Aldrich (Steinheim, Germany). Bis(monoacylglycero)phosphate (S,R isomer) (BMP 18:1/18:1) was obtained from Avanti Polar Lipids (Alabaster, AL, USA).

Acetonitrile and methanol (HPLC gradient grade) were purchased from VWR International GmbH (Darmstadt, Germany). Methyl *tert*-butyl ether (M*t*BE) was delivered by Sigma-Aldrich (Taufkirchen, Germany). Ammonium acetate (≥99.99%) and acetic acid (≥99.99%) were obtained from Fluka (Darmstadt, Germany). Water was purified by a Milli-Q Academic-System (18.2 MΩ cm; 0.2 μm filter; Millipore, Molsheim, France). All chemicals were used as received.

Lipid extracts were filtered by syringe filters from Pall Life Sciences (13 mm; 0.2 μm; PTFE membrane), which were purchased from VWR International GmbH (Darmstadt, Germany).

### 2.2. MCF-7/NeuT cell system, cultivation and lipid extraction

The MCF-7 cell line is widely used in breast cancer studies. Original cells were first isolated in 1970 from breast tissue of a 69-year old Caucasian woman. The MCF-7/NeuT cell line was established by Trost et al. in 2005 to enable conditional expression of NeuT [43]. NeuT is a constitutively active oncogenic variant of the tyrosine kinase ErbB2 receptor. In the MCF-7/NeuT cell line NeuT expression is controlled by a Tet-On-system, which allows tetracycline or derivates such as doxycycline (dox) dependent expression of NeuT. As a consequence of the overexpression of NeuT, MCF-7/NeuT cells undergo premature senescence.

MCF-7/NeuT cell line was cultured in DMEM including glucose (4.5 g/L) and supplemented with tetracycline-free FCS (10%) as described by Trost et al. [43]. Incubation time of dox-treated cells was 7d. Subsequently, untreated and dox-treated MCF-7/NeuT cells were washed with 1× PBS and detached by trypsinization. The reaction was stopped by adding fresh full media. Casy-Technology by Roche Innovatis AG (Bielefeld, Germany) was used for cell number determination. Two samples per condition were pooled to achieve aliquots of $4.5 \times 10^7$ untreated cells and $4.6 \times 10^7$ dox-treated cells

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