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A Comprehensive Analytical Strategy for the Simultaneous Detection and Semi-Quantification of Anionic Phospholipid Species in Plants

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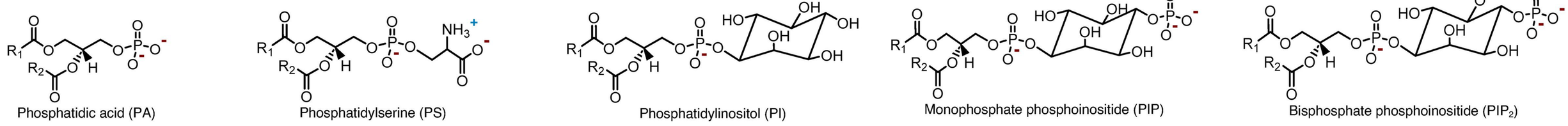
1. Introduction

Anionic phospholipids (PS, PA, PI, PIPs) are low-abundance lipids with key roles in plant cell signaling, membrane trafficking, and differentiation processes. They undergo rapid metabolism in plant and can transiently accumulate at specific cellular or organ sites in response to physiological or environmental cues. Because even minor modifications in their composition can strongly impact biological functions, the development of a sensitive and optimized analytical method for their accurate detection and quantification from plant samples is essential. Although other methods, such as thin-layer chromatography coupled with gas chromatography, have been used for their analysis, these techniques do not allow a precise, sensitive, and accurate quantification of all anionic phospholipid molecules in a one-shot analysis.

2. Objectives

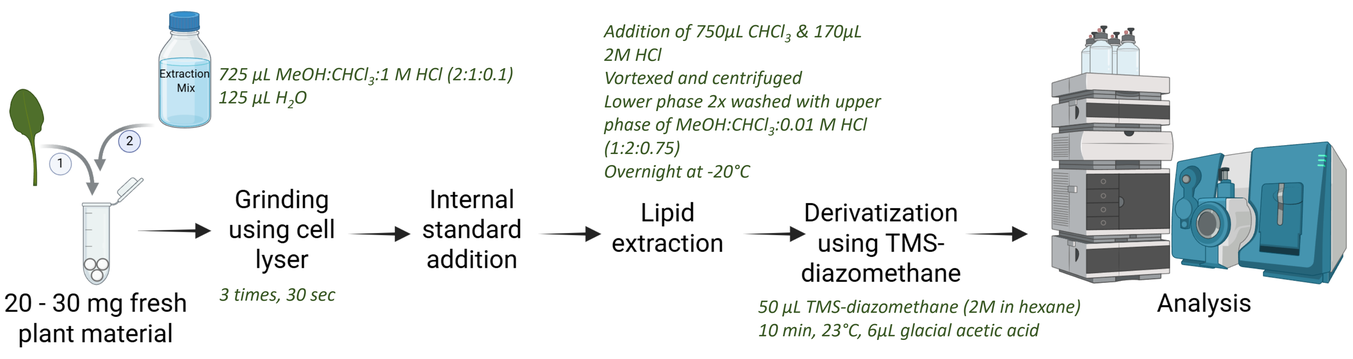
The objective of the present study was to develop a method based on high-performance liquid chromatography (HPLC) combined with two-dimensional mass spectrometry (MS^2) in multiple reaction monitoring (MRM) mode, that enables the simultaneous detection and semi-quantification of all molecular species and classes of anionic phospholipids in a single run.

This approach incorporates a methylation derivatization step in order to highly improve ionization efficiency, peak separation, resolution, and limits of detection and quantification, particularly for PA and PS species.



Structure of the anionic phospholipid classes

3. Sample Preparation and Analysis



Washing 2x with 700 µL of the upper phase of a mix of MeOH:$CHCl_3$:H2O (1:2:0.75; by volume), vortex and centrifugation (1500 g/ 3 min).
Addition of 100 µL MeOH:H_2O (9:1; by volume) to the organic phases, concentration of the samples under a gentle flow of air until only a drop remained.
Addition of 80 µL Methanol, ultrasounds for 1 min, addition of 20 µL water, 1 more min of sonication.
Transfer to HPLC vials for analysis.

Analysis of methylated and unmethylated anionic phospholipids using a HPLC system (1290 Infinity II, Agilent) coupled to a QTRAP 6500 mass spectrometer (Sciex).
Chromatographic separation on a reverse-phase C18 column (SUPELCOSILTM ABZ PLUS; 10 cm x 2.1 mm, 3 µm) using methanol: water (3:2) as solvent A and isopropanol: methanol (4:1) as solvent B at a flow rate of 0.2 mL/min. All solvents are supplemented with 0.1% formic acid and 10 mM ammonium formate.
Injection of 10 µL of samples; with the following percentage of solvent B during the elution gradient : 0– 20 min, 45%; 40 min, 60%; 50 min, 80%; 52 min, 100%; 61 min, 100%; 61.1–68 min, 45%.
The column temperature is kept at 40°C. Mass spectrometry analysis was performed in the positive ionization mode.

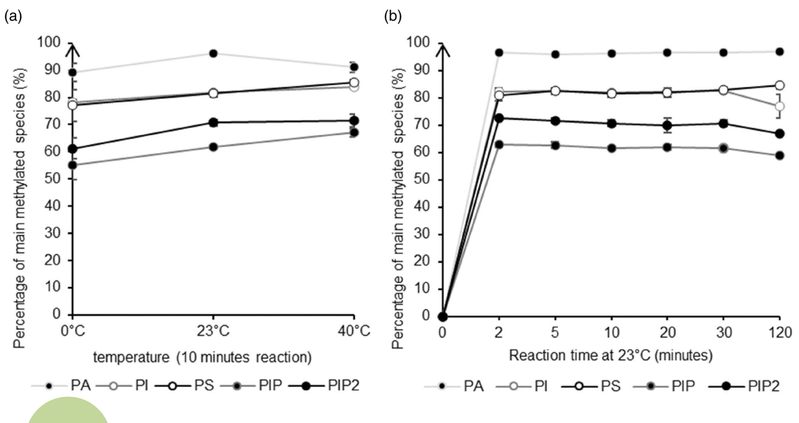
4. Results & Discussion

a Optimization of HPLC and MS parameters for the profiling of methylated anionic phospholipids

Lipid class	Number of additional methyls	Precursor ion	Fragment ion	Declustering potential (V)	Collision Energy (V)	Collision cell exit potential (V)
Phosphatidic acid (PA)	2	$[M + NH_4]^+$	$[M + H\text{-}126]^+$	10	26	19
Phosphatidylinositol (PI)	1	$[M + NH_4]^+$	$[M + H\text{-}274]^+$	10	31	25
Phosphatidylserine (PS)	2	$[M + H]^+$	$[M + H\text{-}213]^+$	10	34	24
Phosphatidylinositol monophosphate (PIP)	3	$[M + H]^+$	$[M + H\text{-}382]^+$	10	32	24
Phosphatidylinositol bis-phosphate (PIP_2)	5	$[M + H]^+$	$[M + H\text{-}490]^+$	10	45	32

Optimized electrospray ionization—MS/MS analysis parameters for the analysis of methylated anionic phospholipids in the positive mode. *For each anionic phospholipid class, the skeletal structure is presented before and after the methylation reaction; as well as the main precursor ion, the fragmentation pattern, and the optimized conditions for their analysis by mass spectrometry.*

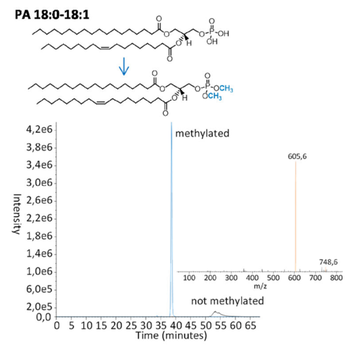
b Optimization of the methylation reaction conditions: evaluation of the impact of time and temperature



Optimization of the methylation reaction conditions. *Impact of the methylation temperature at a constant reaction time of 10 min (a) and of the reaction time at a constant temperature of 23°C (b) on the percentage of the main methylated species for each analyzed class of anionic phospholipids (n = 3). Monomethylated species were considered for PI; bismethylated species were considered for PA and PS. For the analysis of PIP and PIP_2, species methylated 3 and 5 times were, respectively, considered.*

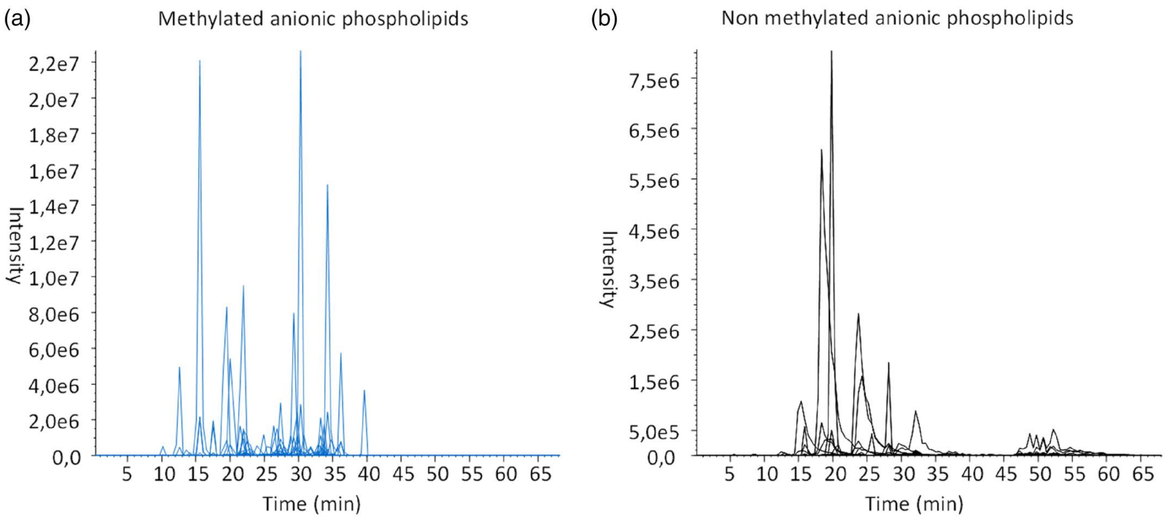
c Analytical performance of the method

Lipid species	Methylated or not	Retention time (min)	LOD (pg / fmol)	LOQ (pg / fmol)	LOQ fold decrease by methylation	Linearity domain (pg)	R^2	Mean % RSD of calibration curve
PA 18:0/18:1	Methylated	38.6	0.19 / 0.27	0.38 / 0.54	128	0.38–1562	0.9956	2.30
	Unmethylated	54.0	12.21 / 17.37	48.83 / 69.46		48.83–12 500	0.9974	8.22
PS 16:0/18:1	Methylated	24.4	3.05 / 4.00	12.21 / 16.02	4	12.21–3125	0.9958	2.46
	Unmethylated	26.4	24.41 / 32.03	48.83 / 64.08		48.83–3125	0.9895	6.26
PS 18:0/18:2	Methylated	27.0	3.05 / 3.87	12.21 / 15.49	4	12.21–3125	0.9979	2.45
	Unmethylated	28.9	24.41 / 30.97	48.83 / 61.96		48.83–3125	0.9888	6.49
PI 16:0/18:1	Methylated	26.1	3.05 / 3.64	6.10 / 7.29	32	6.10–3125	0.9913	5.25
	Unmethylated	23.7	97.66 / 116.67	195.31 / 233.32		195.31–12 500	0.9882	11.54
PI 17:0/14:1	Methylated	15.4	3.05 / 3.84	6.10 / 7.67	32	6.10–3125	0.9973	5.19
	Unmethylated	13.6	97.66 / 122.84	195.31 / 245.67		195.31–12 500	0.9970	10.64
PI4P 17:0/20:4	Methylated	25.0	3.05 / 3.20	6.10 / 6.40	/	6.10–3125	0.9906	4.52
	Unmethylated	/	/	/		/	/	/
PI(4,5)P_2 17:0/20:4	Methylated	24.7	12.21 / 11.82	24.41 / 23.63	/	24.41–3125	0.9878	3.75
	Unmethylated	/	/	/		/	/	/



d Conclusions

The method has been succesfully applied for the determination of the composition of anionic phospholipids in leaves from different plant species (*A. thaliana*; *N. benthamiana*; *Z. mays*), and in different organs of *A. thaliana* (seedlings, roots, cultured cells, plasma membranes). This work establishes a robust analytical platform for exploring how anionic lipid composition is finely regulated during plant development and environmental adaptation, in diverse plant samples.



Chromatographic resolution of the developed method. *Samples containing mixtures of PA, PI, PS, PIP, and PIP_2 standards analyzed with the developed HPLC-MS/MS method following methylation (a) and without methylation (b).*

5. References and funding

[1] Genva, M. et al., The Plant Journal, 2024, 117, 956–971. [2] Genva, M. et al., Bio-protocol, 2025, 15(8): e5282. [3] Bahammou, D. et al., The Plant Journal, 2024, 119, 1570-1595.

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