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RESEARCH ARTICLE

Optimization of chromatographic buffer conditions for the simultaneous analysis of phosphatidylinositol and phosphatidylinositol phosphate species in canola

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The phosphatidylinositols and phosphatidylinositol phosphates are a set of closely related lipids known to influence various cellular functions. Irregular distributions of these molecules have been correlated with the development and progression of multiple diseases, including Alzheimer's, bipolar disorder, and various cancers. As a result, there is continued interest regarding the speciation of these compounds, with specific consideration on how their distribution may differ between healthy and diseased tissue. The comprehensive analysis of these compounds is challenging due to their varied and unique chemical characteristics, and current generalized lipidomics methods have proven unsuitable for phosphatidylinositol analysis and remain incapable of phosphatidylinositol phosphate analysis. Here we improved upon current methods by enabling the sensitive and simultaneous analysis of phosphatidylinositol and phosphatidylinositol phosphate species, whilst enhancing their characterization through chromatographic resolution between isomeric species. A 1 mM ammonium bicarbonate and ammonia buffer was determined optimal for this goal, enabling the identification of 148 phosphatidylinositide species, including 23 lyso-phosphatidylinositols, 51 phosphatidylinositols, 59 oxidized-phosphatidylinositols, and 15 phosphatidylinositol phosphates. As a result of this analysis, four distinct canola cultivars were differentiated based exclusively on their unique phosphatidylinositide-lipidome, indicating analyses of this type may be of use when considering the development and progression of the disease through lipidomic profiles.

KEYWORDS
characterization, chromatography, glycerophospholipids, lipidomics, mass spectrometry, oxidized lipids, phosphatidylinositol, phosphoinositides

ARTICLE RELATED ABBREVIATIONS: DDA, data dependant analysis; MeOH, methanol; PCA, principal component analysis; PI, phosphatidylinositol; PIP, phosphatidylinositol phosphate.

1 | INTRODUCTION

The phosphatidylinositols (PIs) are a class of biologically important, non-abundant phospholipids, distinguished by a common *myo*-inositol headgroup. These lipids, alongside their phosphorylated derivatives (i.e., the PI phosphates, or PIPs), are ubiquitous in eukaryotic life and aid in coordinating various key cellular processes, including signal transduction, membrane trafficking, and cell proliferation [1–6]. As a result of their widespread activities, aberrations that influence a cell's ability to regulate these lipids can result in disease, and abnormal levels are often noted as a distinguishing feature in Alzheimer's disease [2, 7, 8], Bipolar disorder [7, 8] and cancer [2, 7–10]. Such lapses in regulation can likewise result in their irregular molecular distribution, permitting analyses to reveal the development of such pathologies. Hence, continual interest in the activity of these molecules motivates their in-depth characterization [1, 3, 6, 11, 12]. Unfortunately, conventional lipidomic methods have proven inadequate when considering these species, as their physical and chemical properties are often prohibitive to their analysis [13].

Current lipidomic characterizations often employ RPLC-ESI-MS/MS, as the combination of these techniques enables a highly selective and sensitive analysis [14, 15]. Lipidomic methods based on this format regularly pursue the rapid and simultaneous characterization of numerous classes at once and have been optimized for purposes of high throughput and sample fingerprinting [14, 16]. So-called comprehensive methods have rapidly become the standard approach in lipidomic analysis and are likewise employed in characterizations where only a single lipid class is considered [14, 16–18]. However, these generalized protocols often compromise conditions favorable for individual classes to achieve maximum lipidome coverage and, as a result, may prove non-conducive to the analysis of certain lipid classes. PIs, for example, exhibit suppressed ionization under these generalized conditions, which likewise achieve poor chromatographic resolution between isomers of this class, despite readily doing so for other lipid classes [19–23]. In extreme cases, whole lipid classes may be overlooked despite the potential for their presence in a sample. Such is the fate of the PIPs, which are regularly excluded from these characterizations due to their high polarity, poor chromatographic performance, poor ionization efficiency, their propensity to chelate to various surfaces, and their requirement for specialized extraction conditions [24, 25]. Their analysis has instead been relegated to alternative and specialized protocols, resulting in a disconnect between PI and PIP characterizations, despite their biological connection. Hence, characterizations that consider these lipids are disjointed and often lack the depth achieved with other classes, and there are no alternative methods that address these complications.

We previously demonstrated that modifications made to the mobile phase buffers used in current generalized protocols are sufficient to bias conditions to favor the analysis of PIs, allowing for their characterization with greater depth [23]. However, these conditions remained unsuitable for the analysis of PIPs. Therefore, this investigation aims to expand upon our previous work and enhance the characterization of PI species and their phosphorylated derivatives by permitting their simultaneous analysis.

In addition to those advances previously made in the analysis of PIs, various sources throughout the literature have suggested a number of other variables which may be leveraged to pursue this goal. Experimental work regarding the influence of mobile phase pH and solvent composition has suggested that an alkaline and acetonitrile-based mobile phase may be sufficient to enhance the chromatographic retention, resolution, and ionization efficiency for these lipid classes [18, 23, 26–28]. Furthermore, it is suggested that the chelation behavior and poor chromatographic performance of PIPs may be addressed through the use of certain specialized stationary phase technologies [29] and mobile phase additives [30], circumventing the previous necessity of ion-pairing agents and their potential for instrument contamination [27, 31, 32].

Based on the abovementioned conditions, a new chromatographic method was optimized and applied to the characterization of PI species present across four cultivars of canola. The results were then compared against those of our previous study [23]. Canola was again selected as a matrix due to its proportionally high content of PIs (representing 11%–18% of phospholipids) and unique fatty acid profile [33, 34]. Finally, the PI and PIP species identified in each cultivar were then used in the principal component analysis (PCA) to determine whether the distribution of molecular species present was specific enough to permit their differentiation. Such a result may indicate the utility of these conditions in distinguishing the molecular changes which occur in the development of pathology.

2 | MATERIALS AND METHODS

2.1 | Materials and standards

ACN and Methanol (MeOH) were of LC/MS grade, Honeywell B&J brand, purchased from ChemSupply Australia. Chloroform ($CHCl_3$) was of LC grade, LiChrosolv brand, purchased from Sigma-Aldrich (Castle Hill, Australia). Glacial acetic acid of 98% purity or greater and 12 M Hydrochloric acid (12 M HCl) were both purchased from Sigma-Aldrich. Ammonium acetate, ammonium

hydroxide, ammonium fluoride, and ammonium bicarbonate (ammonium hydrogen carbonate) were also purchased from Sigma-Aldrich and were of analytical reagent grade or higher. Ultra-pure water (18.2 MΩ cm) was sourced from an in-lab water purification system (Sartorius), henceforth referred to as H_2O. Phospholipid standards (17:0-14:1 PI, 18:1-18:1 PI, 18:0-20:4 PI, 18:1-18:1 PI(3)P, 18:1-18:1 PI(5)P) were purchased from Avanti Polar Lipids Inc. Oasis WAX 1 cc cartridges (30 mg stationary phase), and the ACQUITY Premier CSH C18 column (150 x 2.1 mm, 1.7 μm) were purchased from Waters Australia Pty Ltd (Rydalmere, Australia).

Four cultivars of canola seed (44Y90CL, 45Y93CL, 45T03TT, and NuseedDiamond) were provided for analysis by the Department of Primary Industries NSW, Wagga Wagga.

2.2 | Sample preparation

Canola extracts were prepared based on an acidified chloroform extraction protocol which has elsewhere been used for the analysis of phosphatidylinositide species [27]. Briefly, 500 μl of 1 M HCl and 500 μl of MeOH containing 0.2 ppm 17:0-14:1 PI as internal standard was added to a mass of 20 mg homogenized canola seed in a 2 ml Eppendorf tube. After 10 s of vortexing and 30 s of sonication, the phase split was initiated by adding 500 μl of $CHCl_3$ to the sample. The sample was vortexed for an additional 10 s and then left on ice for 5 min. After 1 min of centrifugation at 1000 g, the resulting chloroform layer was removed (approximately 500 μl), diluted with 500 μl of MeOH, and then applied directly to a 30 mg Oasis WAX cartridge that had been primed prior by passing through 2 ml of MeOH, followed by 2 ml of glacial acetic acid: MeOH (1:4) and finally, 2 ml of $CHCl_3$: MeOH (1:1). After the application of the sample, fractions containing both neutral and cationic lipids were eluted with an additional application of 2 ml of $CHCl_3$: MeOH (1:1). Finally, anionic lipids were eluted and collected from the cartridge using 2 ml of 12 M HCl: $CHCl_3$: MeOH (1:6:6). After collection, the acidified solution was immediately washed with the addition of 1 ml of UP water, whereafter the chloroform layer was once again collected and dried under a flow of N_2, before reconstitution in a solution of 50% ACN containing 2 mM ammonium bicarbonate and 2 mM ammonia. After reconstitution, samples were applied directly for analysis. A blank extract, which contained only the internal standard, was prepared alongside five replicate extractions per cultivar. A quality control sample was prepared for each cultivar by pooling together the five replicate extracts. Analysis occurred on the same day of the extraction to limit the possibility of sample degradation.

2.3 | Instrumentation

Mass spectrometric analysis was conducted using a Thermo Fisher Q Exactive Plus mass spectrometry system in Top-N data-dependant analysis mode. An MS^1 scan accumulated from 500 to 1000 *m/z* for a maximum injection time of 80 ms, and a mass resolution of 35 000. MS^2 scans targeted the eight most intense ions for a maximum injection time of 60 ms each, with an isolation window of 1.5 *m/z*, fixed first mass of 120.0 *m/z*, and normalized collision energy of 30. The mass resolution for these scans was 17 500. The ESI ion source was held at position 'B'; the sheath gas, aux gas, and sweep gas were 50, 10, and 0 arbitrary units, respectively. Electrospray voltage was −2.5 kV and capillary and aux gas temperatures were at 350 and 300°C, respectively. The S-lens RF level was at 70 arbitrary units.

Chromatography was conducted using an ACQUITY Premier CSH C18 column (15 cm x 2.1 mm), and Thermo Fisher Vanquish quaternary pump system. The optimized chromatographic conditions are as follows:

Flow was maintained at 0.5 ml/min, with a column temperature of 55°C. Mobile phase A consisted of 1 mM ammonium bicarbonate and 1 mM ammonia in H_2O, whereas B consisted of 95:5 ACN: H_2O. Gradient conditions began at 40% B and elevated to 50% B at 0.25 min, followed by 70% B at 6 min, 82% B at 12 min, and 100% B at 14 min. For wash and equilibration, the gradient was then held at 100% B until 18 min, where it then immediately reverted back to 40% B until 25 min.

3 | RESULTS AND DISCUSSION

3.1 | Method development

Initial experimentation was designed to compare the chromatographic performance imparted by certain buffers in terms of their utility for both PI and PIP analysis. The buffer additives (ammonium acetate, ammonium fluoride, ammonium bicarbonate, and ammonium bicarbonate with added ammonia) were selected based on previous experimentation, which suggested their suitability both for PIs and the analysis of phosphorylated compounds [16, 23, 30]. These buffers were added to mobile phases A (H_2O + 1 mM buffer) and B (95:5 Acetonitrile: H_2O + 1 mM buffer) to utilize the favorable conditions imparted by acetonitrile whilst eliminating the potential for buffer precipitation through the removal of non-aqueous conditions. Additionally, an ACQUITY Premier CSH C18 column was chosen for these tests as the stationary phase due to its advertised improved chromatographic performance in separating phosphorylated compounds [29]. The column

TABLE 1 A comparison of the measured buffer pHs (aqueous), pKbs, observed peak retention times (RT, min), and baseline widths (W, min) for standards A (PI 18:1_18:1), B (PI 18:0_20:4) and C (PI(3)P 18:1_18:1). Contrary to the expectations set out by the literature, the values for peak width for compound C, and values for retention time for all compounds appeared to vary independently of either pH or pKb.

BUFFER	PH	PKB	RT (A)	RT (B)	RT (C)	W (A)	W (B)	W (C)
Am. Acetate	7.11	9.25	28.32	28.33	23.70	0.25	0.19	1.19
Am. Fluoride	6.03	10.80	26.51	26.57	20.21	0.25	0.20	0.56
Am. Bicarbonate	8.13	7.65	24.70	24.80	17.49	0.28	0.20	0.32
Am. Carbonate	9.11	3.70	24.95	25.04	17.62	0.27	0.18	0.32

temperature was maintained at 45°C. The flow rate was held at 0.4 ml/min over a linear gradient that incremented from 30% to 100% B in 28 min, which was then held at 100% B for an additional 5 min before re-equilibration. A 1 ppm solution of the standards PI(3)P 18:1_18:1, PI 18:1_18:1, and PI 18:0_20:4 in MeOH was prepared to examine the chromatographic performance of PIPs and PIs simultaneously. In addition, the PI species selected were highly hydrophobic and would help determine whether the chosen solvent conditions were sufficient for their elution within a reasonable time frame. Triplicate injections of 1 μl were used to ensure retention time stability throughout the various conditions tested.

Figure 1 demonstrates that the peak shapes and retention times observed for the selected standards varied considerably with the buffers evaluated during the method development phase. Interestingly, all conditions produced suitable peak shapes for the PIs (Figure 1A,B), but the addition of ammonia to the ammonium bicarbonate buffer was necessary to maximize the chromatographic performance of the PIP standard (Figure 1C). This suggested a potential relationship between the mobile phase pH and the chromatographic performance of PIPs, which is consistent with the literature on the chromatography of phosphorylated compounds [26]. Additionally, changes in retention time such as those observed in Figure 1 have previously been related to the pKb of the buffer anion [28]. However, as shown by the data in Table 1, peak widths and retention time produced by the buffers did not correlate with either buffer pH, or the anions pKb. This, rather, suggests the presence of an alternate or competing mechanism relating the buffer to chromatographic performance, perhaps related to each anion's ability to chelate, ion pair, or position in the Hofmeister series. However, these suggestions remain purely speculative and require further evaluation.

In terms of their suitability for analysis, we assessed the lower retention times produced by the ammonium bicarbonate and ammonium bicarbonate with added ammonia buffers to be conducive to the analysis of PIs, as under the ammonium acetate conditions, elution of the chosen standards occurred at approximately 99% mobile phase B. Under these conditions, the mobile phase may prove insufficient to elute more hydrophobic species within a reasonable timeframe, if at all. In comparison, these standards eluted at approximately 91% mobile phase B with both ammonium bicarbonate and carbonate buffers. In addition, despite the reduction in retention time, a clear resolution was maintained between the tail regio-isomers of the PI 18:0_20:4 standard. A slight decline in peak intensity is also observed in this order and may result from the retention time shift, as the differences in the mobile phase composition at the point of elution can influence desolvation and nebulization [35]. However, our previous work suggested that the peak intensities observed under the ammonium fluoride buffer would far outperform those measured with ammonium acetate, yet this theme is not followed here [23]. The differences in ionization efficiency previously observed may, therefore, be reliant on the mobile phase composition used in our previous study, namely either due to the presence of isopropanol in the mobile phase or the more aqueous conditions at the point of elution. In favor of the latter case, a marked increase in intensity is observed for the PIP standard in Figure 1C when compared to the ammonium acetate conditions; this, however, may exclusively be the product of the increased chromatographic performance. A final increment in peak intensity is then observed with the ammonium bicarbonate with added ammonia buffer, potentially as the alkaline conditions may enhance ionization efficiency.

As a result of this process, the conditions of 1 mM ammonium bicarbonate with 1 mM ammonia were deemed satisfactory for the simultaneous analysis of PIP and PI species, and the method was further optimized for purposes of rapid sample throughput. For this optimization process, a second standard solution containing 1 ppm PI(3)P 18:1_18:1, PI(5)P 18:1_18:1, PI 18:1_18:1, and PI 18:0_20:4 in MeOH was prepared to observe whether the resolution could be achieved between both PIP headgroup isomers and PI tail regio-isomers. This development process saw the elimination of buffer from mobile phase B, which was highly organic and poorly solubilized the polar ammonium bicarbonate crystals. Not only did this further reduce the risk of buffer precipitation, but it comparatively increased the elution strength of mobile phase B, reducing the likelihood of exceptionally hydrophobic species carrying over between injections, and prevented

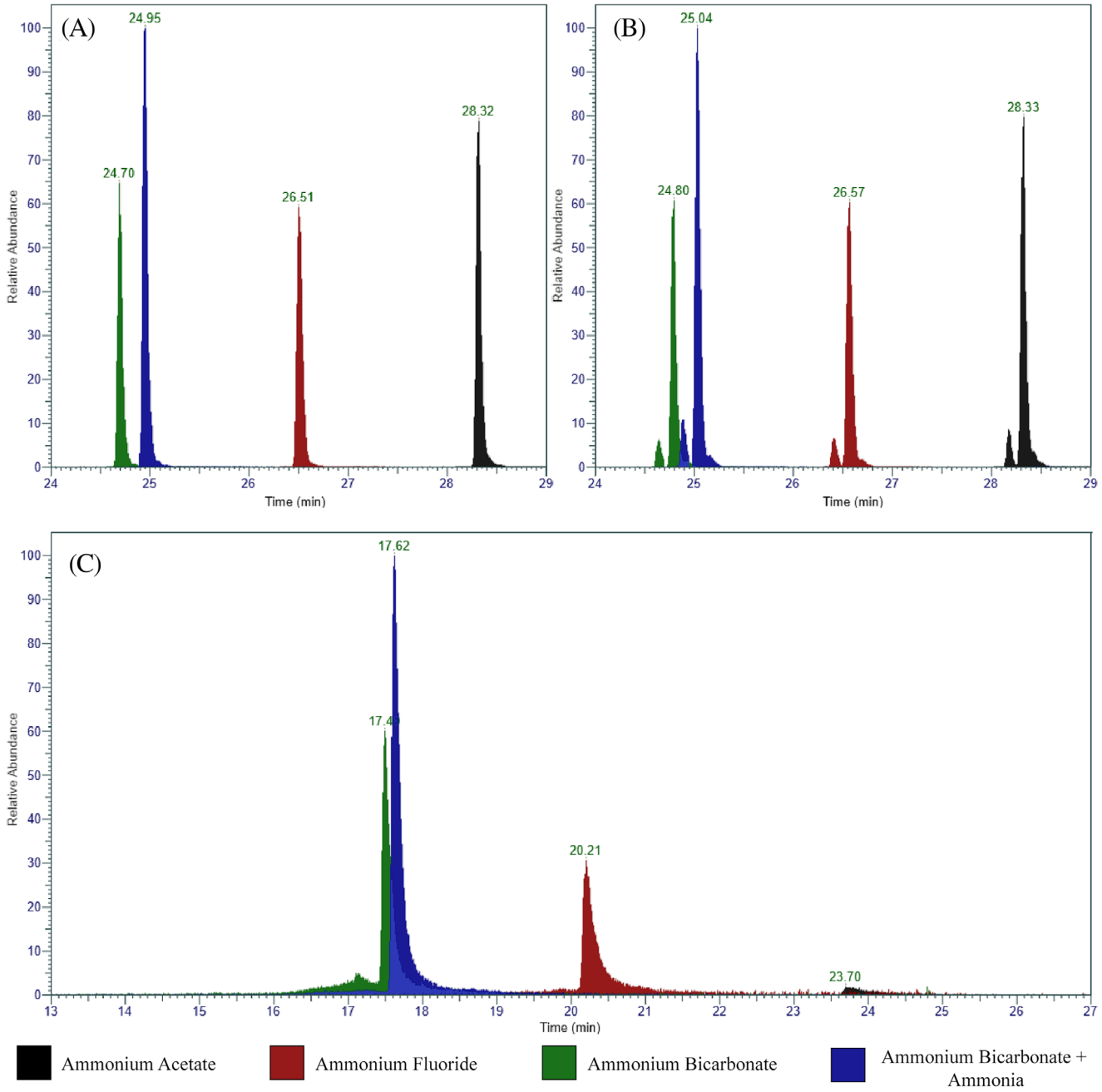

FIGURE 1 Extracted ion chromatograms (EICs) taken from the analysis of a mixture of standards containing: (A) Phosphatidylinositols (PI) 18:1_18:1, (B) PI 18:0_20:4 and (C) PI(3)P 18:1_18:1. The *m/z* values for the [M-H]- ions which correspond to each of these species are as follows: (A) 861.5499, (B) 885.5499 and (C) 941.5162. Four overlayed EICs are displayed per standard, displaying the differing chromatographic behaviors observed with respect to the buffers examined. The four buffers assessed here were ammonium acetate, ammonium fluoride, ammonium bicarbonate, and ammonium bicarbonate with equal parts added ammonia. The ammonium acetate buffer provided sufficient chromatography for the PI standards (A and B), but was unacceptable for the phosphatidylinositol phosphate (PIP) standard (C). A shift in retention was observed for both the PI and PIP standards in the sequential ammonium fluoride and ammonium bicarbonate tests, while the peak shape of the PIP standard improved considerably. The addition of ammonia to the ammonium bicarbonate buffer further improved peak shapes and intensities for all standards considered.

TABLE 2 The precision of peak area measurements throughout and beyond the calibration range. The values provided here are in terms of %RSD, calculated using three repeat measurements per concentration. The precision for peak area measurements remained high from between 5 and 500 ppb, though rapidly declined over measurements less than 1 ppb in concentration. The LOD values provided in Table 3 were estimated by determining the concentration for which %RSD values approximately equaled 25%.

Standard	100 ppt	250 ppt	500 ppt	1 ppb	5 ppb	25 ppb	100 ppb	200 ppb	500 ppb
PI (3) P 18:1 18:1	19.09%	25.96%	8.07%	8.63%	5.71%	2.96%	1.12%	1.15%	0.37%
PI 18:1 18:1	13.10%	15.42%	5.64%	6.16%	0.83%	1.58%	1.28%	1.98%	1.28%
PI 18:0 20:4	51.59%	3.98%	8.36%	4.24%	2.41%	1.13%	1.93%	1.92%	2.57%

the mobile phase from increasing in alkalinity with organic composition, which could potentially exceed the columns limit [36]. The final conditions are provided in section 2.3. As shown in Figures 2A,B, a chromatographic resolution could be achieved between both PIP headgroup isomers and PI tail regio-isomers, the latter of which is verified through their unique fragmentation spectra (Figure 2C,D).

3.2 | Method validation

Following the completion of method development, these conditions were then validated for the purpose of untargeted characterization and applied in the analysis of canola PI and PIP species. Injection carry-over was assessed, with particular attention to the PIPs due to their activity as chelating agents, prior to all other validation steps to ensure accurate measurement of detection limits and linear correlation. Various efforts have been employed elsewhere to mitigate PIPs chelating, including the use of a competing chelating agent such as EDTA [25, 27]. Therefore, a wash solution of 50% acetonitrile containing 25 μM EDTA was here used to aid in mitigating carry-over. In these tests, 2 μl injections of the same 1 ppm PI(3)P 18:1_18:1, PI(5)P 18:1_18:1, PI 18:1_18:1, and PI 18:0_20:4 solution was used, followed by a blank injection. The blank injection was then inspected for traces of the standard mix. No carry-over was observed for the PIP standards; likewise, there was no carry-over observed for the PI standards used.

Validation was then continued to determine the peak area precision over the expected sample concentration range; furthermore, the limits of detection, calibration curve linearity, and retention time precision were also evaluated. These values are summarized in Tables 2 and 3. Values for peak area precision were calculated as a %RSD value over three repeated injections and would be used to estimate the LODs, where the %RSD approximated 25%. As shown in Table 3, the LODs were calculated to be in the range of a few hundred ppt, and the linearity from 5 to 500 ppb exceeded an R^2 value of 0.99 for all three representative lipids. Finally, retention time variation was measured over 12 injections, equivalent to a period of 5 h, and was calculated to be below 0.3%.

The pH of mobile phase A was measured to ensure that the alkaline buffer used did not exceed the pH limits of the ACQUITY Premier CSH C18 column, which reproducibly measured approximately pH 9.11. This pH was well below the column's limit of pH 11.

3.3 | Canola analysis

Five replicate canola extracts were prepared for each of the four cultivars according to the protocol outlined in section 2.2. Each sample was then analyzed with triplicate injections. Quality control samples were prepared by pooling together the five replicate extracts and analyzed on every 10th injection. Data processing was conducted using MS-DIAL [37] in metabolite mode to permit an in-house spectral library catered specifically for the identification of PIs [38]. All identifications were then manually assessed, normalized against the internal standard, and used in PCA. PCA plots were constructed in Python using sklearn and displayed with pyplot.

As a result of the analysis, a total of 148 PI species were identified across the four cultivars, including 23 Lyso-PIs, 51 PIs, 59 Oxidized-PIs, and 15 PIPs. The identifications are available in Table S1 and an example TIC is provided in Figure 3A, along with a set of representative EICs in Figure 3B which depict the approximate retention times for the observed lipid classes. These identifications represent an over 5-fold increase in number compared to our previous study, which used a generalized lipidomics gradient and observed 14 PI species and 12 PI candidates [23]. This considerable increase in identifications is suggested to have arisen due to a number of compounding influences, such as the inclusion of SPE in the extraction process reducing potential matrix effects, and the improved suitability of the analysis method favoring the chromatography and ionization of these species. However, despite the chromatographic conditions being optimized to permit their analysis, the largest increase in identifications did not come from the PIPs but rather from a subset of the PIs with degrees of oxidation. These identified species differed in fatty acid chain length compared to the non-oxidized PI species observed, suggesting that

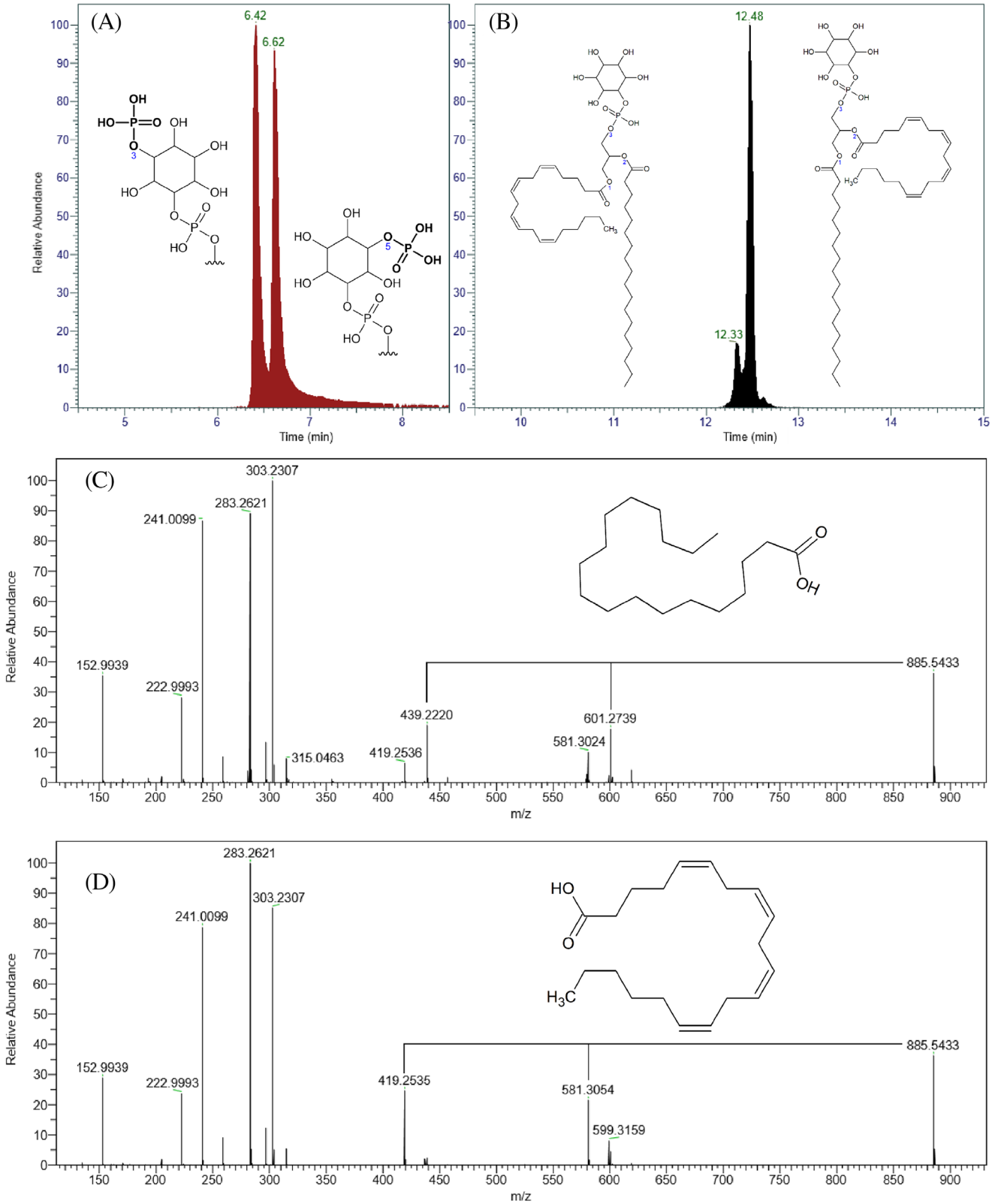

FIGURE 2 The extent of the chromatographic resolution achievable between phosphatidylinositol phosphate (PIP) headgroup isomers (PI(3)P and PI(5)P) (A) and the tail regio-isomers phosphatidylinositols (PI) (PI 20:4/18:0 and PI 18:0/20:4) (B) is shown using the finalized chromatographic conditions. The MS^2 spectra for the two major PI tail regio-isomers, at retention times 12.33 and 12.48, are shown in Figures C and D, respectively. The identity of these isomers could be verified through their unique fragmentation, which corresponded to the identity of the sn2 fatty acid.

TABLE 3 Summarized results from method validation. Limits of detection were determined by sequentially expanding the calibration curve on the lower end until the signal reproducibility varied significantly. This was estimated where the %RSD values for peak area approximately equaled 25%. Numerical values showing the %RSD for all measured concentrations are shown in Table 2. Linearity was determined over the range of 5–500 ppb. Retention time stability was determined over the course of 12 repeat injections, equivalent to 5 h of analysis time.

Standard	*m/z* (Predicted)	LOD	r^2	RT (min)	RT %RSD
PI (3) P 18:1 18:1	941.516182	~250 ppt	0.9981	6.40	0.30
PI 18:1 18:1	861.549852	~100 ppt	0.9993	12.33	0.07
PI 18:0 20:4	885.549852	~250 ppt	0.9992	12.47	0.07

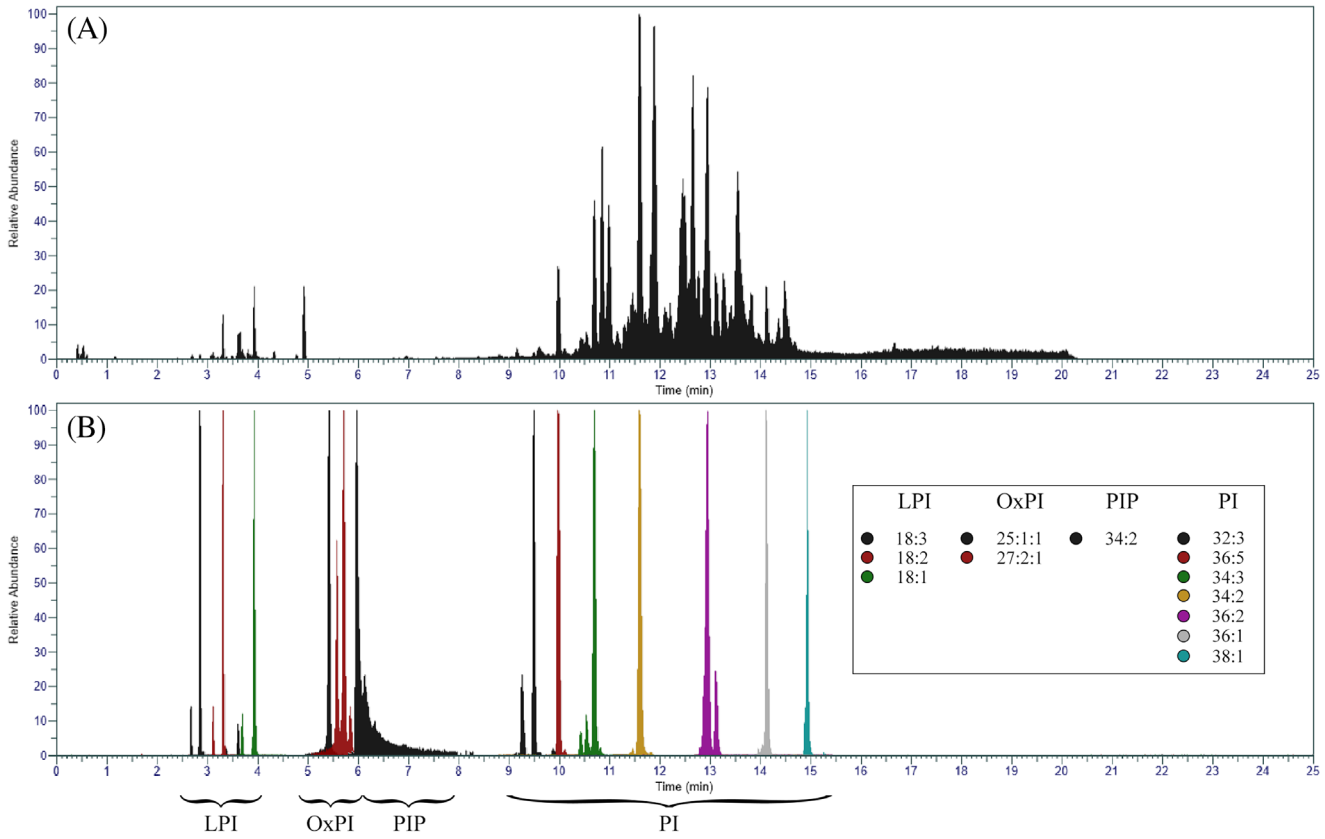

FIGURE 3 (A) provides an example of TIC observed for one of the canola extracts. (B) provides an overlayed set of extracted ion chromatograms (EICs) depicting lyso phosphatidylinositols (PI) species, which generally appeared to elute between 2 and 5 min, phosphatidylinositol phosphate (PIP) species, which elute between 5 and 9 min, oxidized PI species from 5 min onward, and PI species from 9 min onward. All of the overlayed EICs are shown on independent scales.

they were unique and did not originate from sample oxidation during the extraction process. The exact form of oxidation was not determined; however, it is likely that they comprise hydroxy-functionalized fatty acids, as they are a well-known constituent of seed oils and have been observed previously as a minor constituent [39, 40]. The majority of these species shared retention times approximate to those of the PIPs and likewise exhibited diffuse peaks, suggesting that they were also highly polar and may exhibit similar chelation behavior. This is consistent with the chromatographic behavior of oxidized PIs observed elsewhere [41]. Two example EICs of oxidized PI species are provided in Figure 4.

Additionally, 68 of the identified species shared their precursor mass with at least one other identification and thus represented some form of lipid isomer. In comparison to our previous analysis, where only six co-eluting isomeric species were observed due to the presence of unique fatty acid fragments, the present method additionally resolved isomeric species that differed in sn-position, or double bond arrangement, as revealed by MS/MS analysis. The observation of these additional species may have

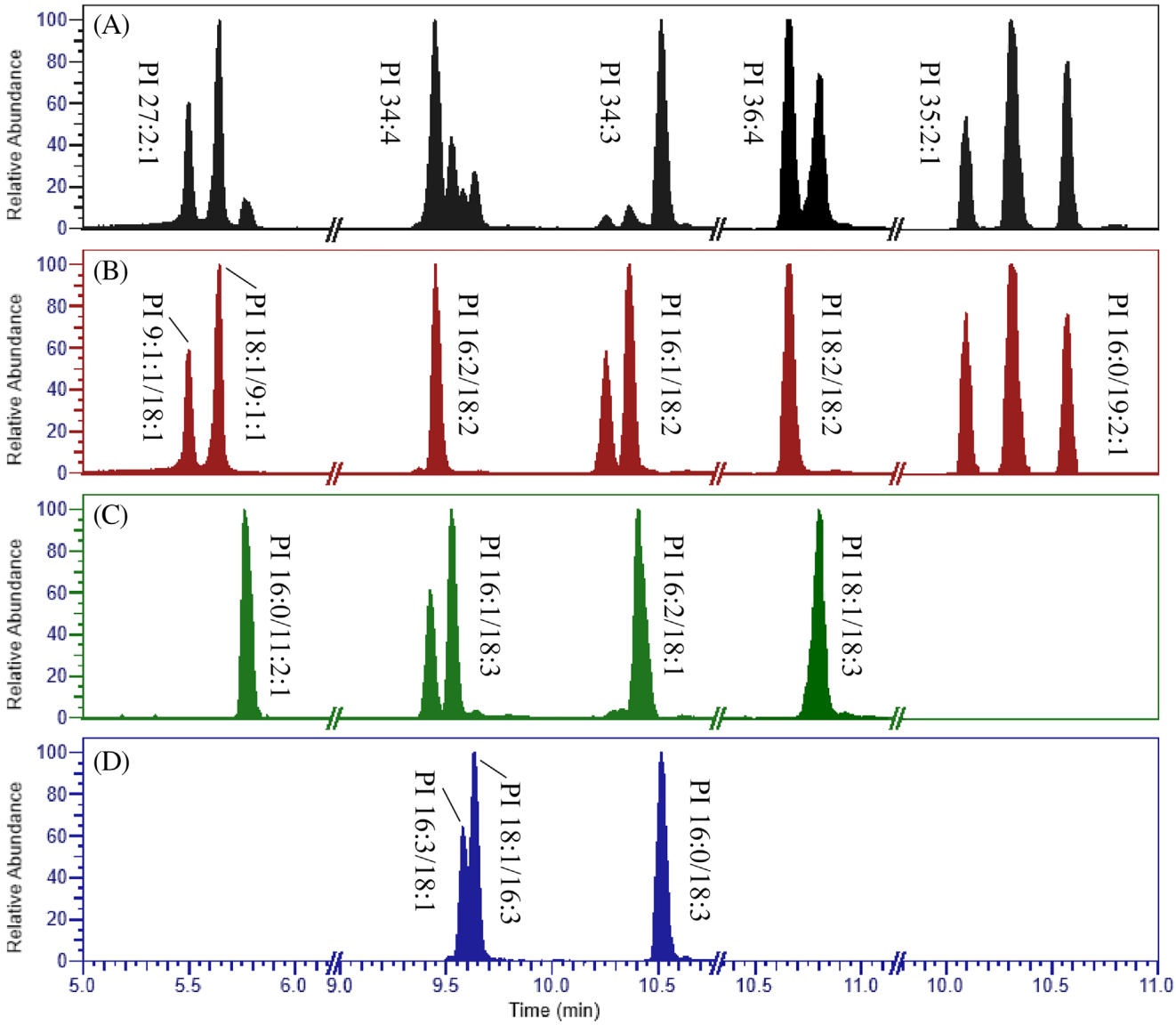

FIGURE 4 EICs for various phosphatidylinositols (PI) species depicting the chromatographic resolution achieved between both tail isomers, tail regio-isomers, and double-bond isomers. In black (A), the extracted ion chromatograms (EICs) for five masses are provided (*m/z* 751.4027, 829.4899, 831.5006, 857.5229, and 861.5309), where each corresponds to a PI species (PI 27:2:1, PI 34:4, PI 34:3, PI 36:4 and PI 35:2:1, respectively). The subsequent red (B), green (C), and blue (D) chromatograms reveal the extent to which isomers may be resolved. For each mass, separation on the horizontal axis represents the resolution between both regio-isomers and double-bond isomers, whereas separation on the vertical axis represents a set of tail isomers.

arisen due to an improvement in ionization conditions; however, an improvement in chromatographic conditions is also suspected to play a role. The solvents used in our previous study were based on those used in generalized lipidomic characterizations and included isopropanol to elute highly hydrophobic lipid species such as triacylglycerols. The removal of isopropanol from the present mobile phase, therefore, sacrificed maximum elution strength in favor of improved selectivity. Additionally, it is suspected that protic solvents such as MeOH or isopropanol may enable accelerated diffusion, allowing their removal to reduce peak broadening. Example EICs for some of these isomers are provided in Figure 4, which depicts the extent of the resolution which may be achieved between them. However, whilst these conditions aided in the resolution between these species, identification was only possible regarding fatty acid combination and sn-position, as the double bond arrangement was not represented in the MS^2 spectra. Furthermore, due to these species still being closely related in a structural sense, many of these species were incompletely resolved; as shown in Figure 4, some EICs display shouldering or broad peaks where isomers

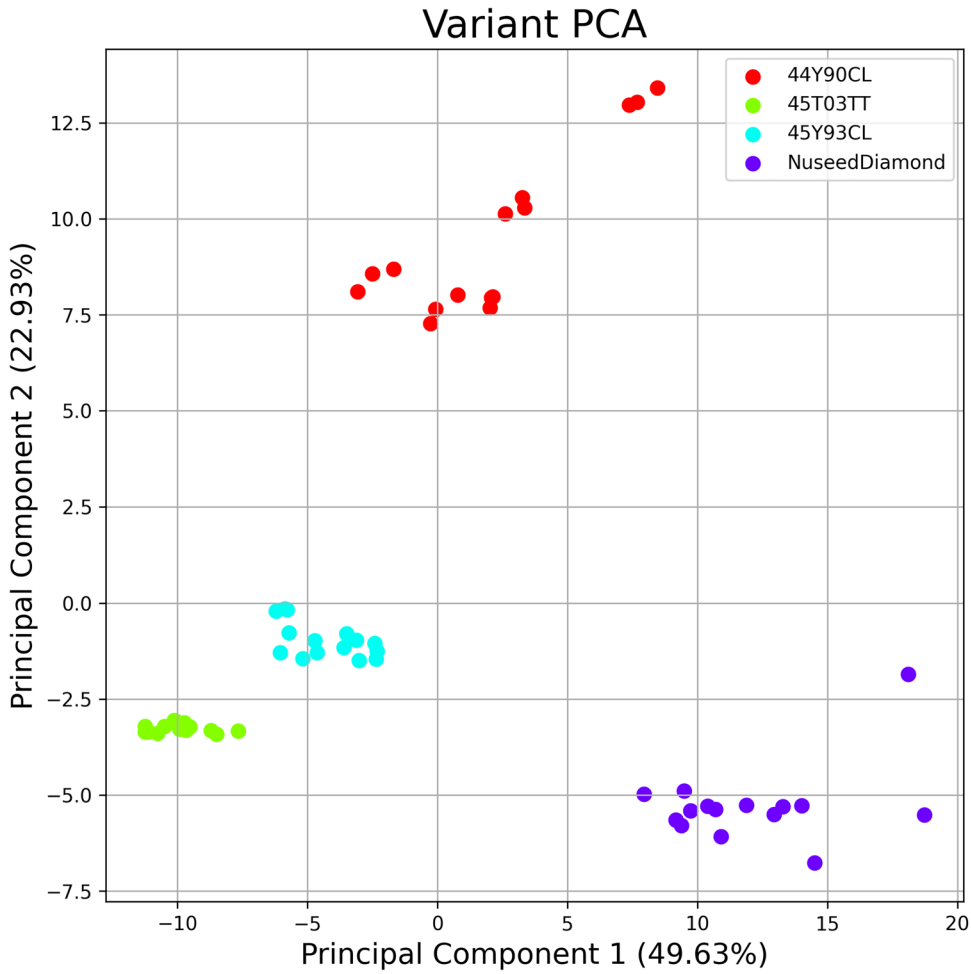

FIGURE 5 A principal component analysis plot of the four canola cultivars examined in the experimental (44Y90CL, 45T03TT, 45Y93CL, and Nuseed Diamond). The principal component analysis (PCA) was conducted in Python using sklearn and displayed via pyplot. Only the identified Lyso-PI, PI, Oxidized-PI, and PIP species were used as input. As a result of the PCA, four distinct clusters were produced, each corresponding to a single cultivar.

partially co-eluted with one another. Such compounds thus represent an additional challenge in the pursuit of comprehensive characterization.

The double bond isomer co-elution issue is not unique to this analysis but represents a challenge observed throughout lipidomics in general. Current alternate approaches enable some resolution between these forms of isomers and may therefore allow an analysis of further depth. Ion-mobility-MS-based techniques are one such alternate approach where it is possible to achieve resolution between lipid isomers under certain conditions [42]. However, additional resolution in this regard permits only differentiation and not identification, as the double bond geometry is not represented in the fragmentation spectra. Recent improvements to general ion mobility-MS systems are enabling the generation of unique fragments corresponding to a lipid's double bond isomerism. Online ozonolysis [43] and Paternò–Büchi reactions [44–46] are two alternative approaches that chemically modify lipids at double bond sites to generate these fragments. These techniques require additional hardware or modifications applied directly to the instrument and are thus not readily accessible. Furthermore, due to the chemical modifications, both the precursor mass and fragmentation spectra of target compounds are modified accordingly, requiring the modification of current spectral libraries.

Fortunately, there is a practical distinction between an absolutely comprehensive characterization and one sufficient for the speciation of a matrix. To determine whether the identifications made were sufficient for the differentiation of canola based on the cultivar, a PCA plot was prepared using the normalized peak areas from the data accumulated. The resulting PCA separation is provided in Figure 5, where four distinct groups may be observed, one belonging to each cultivar. As a product of the PCA, the specific lipid species which significantly correlated for or against the formation of the clusters could be determined, and those species are provided in Table S2. Interestingly,

among those lipid species which correlated, nearly all were PI species containing a single 18-carbon chain, with one or two degrees of desaturation, and a second carbon chain that varied significantly in length and desaturation and often contained at least one degree of oxidation. The similarities between these species may indicate that this second variable fatty acid is one of the most significant sources of variation between the examined cultivars, so far as the PI-lipidome is concerned. An ability to differentiate closely related matrices based entirely on their PI-lipidome may serve as a valuable tool to understand the development and progression of certain diseases, specifically due to the variations these pathologies are known to impart on the PI-lipidome [1, 3, 6, 11, 12].

4 | CONCLUDING REMARKS

Current generalized conditions utilized for comprehensive lipidomic analysis have proven insufficient for the characterization of PI species and do not consider PIPs. In this work, we have demonstrated that a series of simple modifications to pre-existing methods is sufficient to bias analysis conditions in favor of PIs whilst enabling the simultaneous analysis of PIPs. It is suggested that the alkaline conditions imparted by the ammonium bicarbonate with ammonia buffer (at pH 9.11) may be partly responsible for the improvement in chromatographic performance due to the comparatively worse chromatography observed under the other tested chelating buffers such as ammonium bicarbonate (at pH 8.13) and ammonium acetate (at pH 7.11).

In total, 148 PI and PIP species were identified across four cultivars of canola, including 23 Lyso-PIs, 51 PIs, 59 Oxidized-PIs, and 15 PIPs. Although the presence of oxidized lipids has previously been reported in seed oils as a minor constituent, it was unexpected that the majority of PI species observed appeared to be oxidized. These species appeared to elute earlier than other PI species with somewhat diffuse peaks, suggesting that they were highly polar and may exhibit some chelation behavior. Additionally, the unique fatty acid content observed for these species suggests that they were not the product of oxidation during the extraction process. The large quantity of these species observed here may suggest that similar species are being under-reported in other studies, perhaps due to inadequate chromatographic conditions.

From the 148 identifications, 68 shared their precursor mass with another potential identification and represented some form of lipid isomer. Whilst lipid regio-isomers may be differentiated due to the presence of unique fragments, the resolution and identification of lipid double-bond isomers remain difficult; however, a future analysis may be able to resolve this by including complementary techniques such as ion-mobility spectrometry.

Despite the issue of co-eluting isomers obscuring some species, a PCA of the canola PI-Lipidome produced a distinct clustering for each cultivar, permitting their differentiation and identifying which species most significantly contributed to the clustering. In the future, analyses of this type may be useful when considering a disease's development and progression.

AUTHOR CONTRIBUTIONS

David Gertner: Conceptualisation, experimental design, performed experiments, analyzed data, wrote the original draft, and edited manuscript.

David Bishop reviewed and edited the manuscript.

Matthew Padula reviewed and edited the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict if interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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