Enhancing Coverage of Phosphatidylinositol Species in Canola Through Specialised Liquid Chromatography-Mass Spectrometry Buffer Conditions

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Abstract

Phosphatidylinositols (PIs) constitute a minor class of phospholipid with wide-spread influence throughout various cellular functions. Monitoring the distribution of these lipids can therefore provide insight as to the state of cellular processes or reveal the development of various pathologies. The speciation of these compounds is often performed either as part of a comprehensive characterisation of lipids, or specifically targeted using the same methods, however, such methods were intended to maximise coverage of lipid classes rather than provide an in-depth analysis of any single class. In the particular case of PIs, the majority of reported molecular diversity is limited to a small proportion of the already minor class, as such the cursory glance enabled by such methods is insufficient. Therefore, this work compared the suitability of both established and novel LC-MS buffers with the aim of maximising the ionisation efficiency of PIs, in an attempt to enhance coverage of the class. Through experimentation, it was determined that a 0.25mM ammonium fluoride buffer provided up to a 6-fold increase in signal intensity, and on average a 38-fold increase in the signal-to-noise ratio. Using these new conditions, 14 PI species, and 12 PI candidates were identified within a dilute lipid extract sourced from canola seed, compared to 0 species identified using the generalised method. As a result, it is suggested that this procedure has yielded the highest number of PI species identifications for a sample of this concentration. Methods which therefore intend to characterise PI species in dilute quantities, such as those extracted from mammalian cells, are henceforth provided with the means to conduct more comprehensive characterisations.

Keywords

Phosphatidylinositol; Characterisation; Lipidomics; Mass Spectrometry; Canola; Ionisation.

Highlights

- Common conditions for lipidomic analysis are unsuitable for analysis of PI species.

- The ionisation efficiency of several buffers were compared.
- Ammonium fluoride outperformed previous conditions for PI species.
- 26 PI species were observed in dilute canola extract.

1. Introduction

Phosphatidylinositols (PIs) constitute a class of phospholipid which are characterised by the presence of a *myo*-inositol ring substituted as the headgroup. Through cellular processes, the *myo*-inositol ring may be additionally phosphorylated at several of its hydroxy sites, giving rise to an additional seven known PI sub-classes (see Figures 1A and 1B) [1, 2]. These additionally phosphorylated PI species (PIPs) play several key roles in various facets of maintaining cellular functions essential for growth and proper development, and thus are highly regulated within cells [1-6]. Aberrations which may interfere with this process of regulation have been identified as a distinguishing feature associated with a number of serious pathologies such as Joubert syndrome [1, 4], Charcot–Marie–Tooth disease [1, 4, 7, 8], Lowe syndrome [1, 4, 7-9], Bipolar disorder [8, 10], Alzheimer's [1, 8, 10], and some cancers [1, 4, 7, 8, 11, 12]. Due to their pervasive nature, these lipids are interesting biomarkers needing identification, characterisation, and quantification; however, various properties, such as the low abundance of these lipids, makes analysis difficult. In the case of the PIP sub-classes, several specific approaches have been developed which enable targeted analysis, such as the use of ion-pairing or derivatising agents to improve chromatography and electrospray ionisation, each with their own compromise [13-18]. However, despite their greater abundance and thus theoretically easier analysis, methods which observe the precursor unphosphorylated PIs are unspecific and are often derived from a set of parameters which have been generalised for comprehensive lipidomic analysis, and thus are not necessarily well suited for this class of compound [10].

Typically, due to their hydrophobicity, reversed phase liquid chromatography (RP-LC) in conjunction with electro-spray ionisation mass spectrometry (ESI-MS) (Collectively referred to as RP-LC-MS) is utilised for the separation and characterisation of lipid species in complex samples [19, 20]. Those generalised conditions which are intended for comprehensive characterisations of lipids in a single chromatographic separation compromise conditions rather than use those which are ideal for any class as to achieve maximum coverage of the lipidome. As a result, these experiments often require concentrated samples, do not resolve certain isobaric species, and enable purview of only those most intense species. It is here argued that due to their unique characteristics, such generalised parameters are particularly insufficient for the analysis of PIs.

PIs are relatively sparsely occurring in comparison to the other phospholipid classes, as a mix of multiple individual PI species they represent 11-18% of canola lecithin and only about 10% of total cellular phospholipids [1, 3, 4]. Additionally, alike other lipid classes, these molecules are observed with a range of slight structural variations which alter their specific physical and chemical properties, and therefore their biological activities and functions [3]. However, a distinctive feature of PIs is that in mammalian cells a significant proportion is biased towards a specific species, namely the 1-stearoyl-2-arachidonoyl form, shown in Figure 1C [1, 3]. It has been suggested that this form may allow for adequate membrane packing, exposing the headgroup for efficient interaction [3]. As a result of this bias, the majority of the reported molecular diversity for this class is limited to a small proportion of an already minor lipid class. However, as these lipids are highly regulated, it is suggested that these minor species are not biologically inconsequential. It is when considering the analysis of these minor species, whether it be for characterisation or observing potential aberrations in their regulation, that the limitations of the generalised parameters inherited from comprehensive lipidomic characterisations become most apparent.

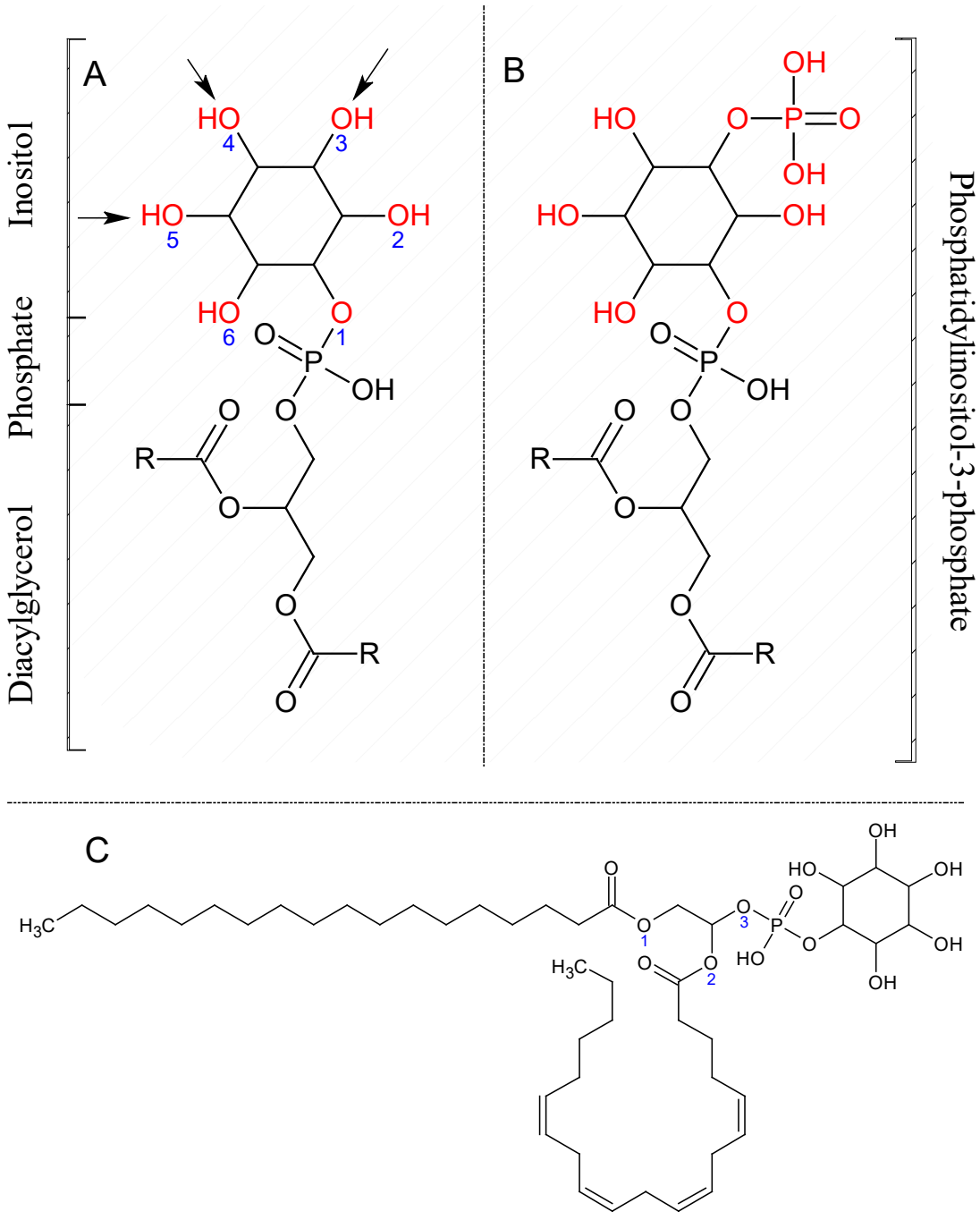

Figure 1. A & B *General structure of phosphatidylinositols and phosphatidylinositol phosphates*. *Hydroxy sites available for additional phosphorylation are marked with an arrow*, **C** *Structure of 1-stearoyl-2-arachidonoyl phosphatidylinositol, the most common PI species in mammalian cells.*

Fortunately, previous work has shown that that these generalised conditions may be biased to improve the analysis of particular classes. For example, Cajka and Fiehn [21] have previously demonstrated through testing a variety of common buffers and acids, that the ionisation efficiency for particular lipid classes could be selectively bolstered. Their results had suggested that a 10 mM ammonium acetate (AmAc) buffer enabled the greatest ionisation efficiency for several lipid classes in negative ESI, including PIs. However, more recently the utility of AmAc has come into question, as conflicting data has suggested otherwise. In a similar assessment of common buffers and acids, Monnin C. *et al*. [22] have shown that the addition of AmAc severely hinders ionisation efficiency for a range of lipid classes,

including PIs, whilst only promoting the ionisation efficiency of phosphatidic acid, ceramides and phosphatidylcholines. It had been their conclusion that for the analysis of lipids in negative ESI, 0.02% acetic acid enabled the greatest ionisation efficiency.

Undoubtedly, for a comprehensive characterisation of PI species, further research is required to assess the validity of AmAc, and to examine potential alternative conditions which may supersede it in ionisation efficiency. An improvement in the ionisation efficiency for these compounds would hence enable the characterisation of the diverse minor species endemic to this class.

In terms of potential substitutes for AmAc, there is some precedent for the use of other buffers such as ammonium hydroxide (AmOH) and more recently ammonium fluoride (AmF). Although their use has been limited, both have been suggested to improve ionisation efficiency for various small molecules [23-26]. As there has only been limited assessment for these buffers in terms of their ability to ionise phospholipids, their utility in comparison to AmAc was measured in this work. Furthermore, a variety of basic modifiers have been used in negative ESI as post column additives to enhance the ionisation efficiency for compounds of interest [27]. Therefore, in similar fashion, a select few would need to be considered for the purpose of further enhancing ionisation efficiency. However, as affirmed elsewhere and iterated in the aforementioned works of Cajka and Feihn [21] and Monnin C. et. al [22], the ionisation efficiency of phospholipids appears to be sensitive to various factors, including the interactions between the lipid headgroup and solvents used [28]. As such, it would be necessary to further explore the influence of the solvents used during chromatography on the efficiency of ionisation of PIs and PIPs.

To assess the degree any improvement in ionisation efficiency would translate into an improvement in analysis, an attempted characterisation of PI species present within a canola extract was conducted using the finalised conditions, after which it was compared to a characterisation performed using the initially suggested 10mM AmAc additive. For the purpose of these characterisations, a canola extract had been elected as a model due to a proportionally high content of PIs (representing 11 – 18% of phospholipids) and unique fatty acid profile, as outlined in a previous characterisation of rapeseed lecithin [29, 30]. Hence it was suspected that a diverse group of relatively intense PI species may be observed without the need for preconcentration.

Thus, the aims of this investigation is to determine the solvent, additive and buffer conditions which would specifically enhance PI detection and quantification through enhancing electrospray ionisation efficiency, and to then determine whether the increase in ionisation efficiency would in fact enhance the characterisation of these lipids.

2. Materials and methods

2.1 Chemicals and standards

Methanol (MeOH), acetonitrile (ACN), and isopropanol (IPA) used were of LC/MS grade, B&J Brand, purchased from Honeywell.

Ultra-pure water (UP) (18.2MΩ-cm) was sourced from an in-lab water purification system (Sartorius).

Phospholipid standards (17:0-14:1 PI, 18:1-18:1 PI, 18:0-20:4 PI, 18:1-18:1 PI(3)P) were purchased from Avanti Polar Lipids Inc.

Ammonium acetate (AmAc), ammonium hydroxide (AmOH) and ammonium fluoride (AmF) were purchased from Sigma-Aldrich and were of analytical reagent grade or higher.

Pyridine, piperidine and *N,N*-diisopropylethylamine were also purchased from Sigma-Aldrich and were of 98% purity or greater.

Canola seed was of an unknown cultivar, purchased from Reptile Direct Australia.

2.2 Standard Solutions

Canola extract was prepared through an MtBE extraction protocol, using 100 mg canola seed which had been homogenised in a ball mill. The resulting extract was then reconstituted into 2 mL MeOH [31].

Briefly, 1.5 mL of MeOH and 5 mL of MtBE was added to the 100 mg sample of homogenised canola seed in a 15 mL falcon tube. The sample was mixed on a rotisserie for an hour and then left to rest for 10 minutes. Next, 1.25 mL of UP water was added, and the sample shaken. The sample was then centrifuged at 1000 g for 10 minutes. The upper layer was then removed and dried under N2 before reconstitution in 2mL of MeOH.

Two standard solutions were prepared for the purpose of conducting both flow injections and chromatographic separations. Standard solution 1 consisted of 1 ppm 17:0-14:1 PI, 18:1-18:1 PI, 18:0-20:4 PI, and 18:1-18:1 PI(3)P in 100% ACN. Standard solution 2 consisted of 1 ppm 17:0-14:1 PI added to a mixture of 50% canola extract and 50% ACN.

Despite the experimentation not targeting additionally phosphorylated PIs, the 18:1-18:1 PI(3)P standard was included within the standard mixture to observe whether the conditions that favoured the analysis PIs likewise produced favourable conditions for their derivatives.

2.3 Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)

LC-MS/MS analysis was conducted using an Agilent 1290 Infinity LC system, coupled to an Agilent 6510 qTOF mass spectrometer. Chromatography was conducted using a Waters Acquity CSH column (1.7um, 135A, 150mm x 2.1mm). Data was acquired under electrospray ionisation (ESI) negative mode with the optimised source parameters as follows; gas temperature was 365 ºC; drying gas (nitrogen), 5L/min; nebuliser gas (nitrogen), 30 psig; capillary voltage, -4kV; fragmentor voltage, 80V; skimmer voltage, 65V. MS1 spectra were collected by accumulating over m/z 500 – 1000 for 0.25s; MS2 spectra were then collected when a precursor ion exceeded 3000 counts, and fragments were observed over m/z 125 – 1000.

Through a series of flow injections, the ideal buffer conditions under which PIs would ionise were to be determined. Buffers and modifiers examined included AmAc, AmOH and AmF (shown in Table 1), as well as piperidine, pyridine and *N,N*-diisopropylethylamine (shown in Table 2) at varying concentrations. During the flow injection experiments only MS1 data was gathered, whereas during the chromatographic experiments, both MS1 and MS2 data were gathered.

After optimisation, results for the new buffer condition were then compared to those results gathered using the AmAc buffer. Four replicate injections were completed using each condition. For each separation, 5uL of standard solution 2 was injected, the column was maintained at 65 ºC, and flow was maintained at 0.5mL / minute. The gradient follows: 0 minutes 15% B, 0 – 2 minutes 30% B, 2 - 2.5 minutes 48% B, 11 – 11.5 minutes 99% B, 11.5 – 12 minutes 99% B, 12 - 12.1 minutes 15% B, 12.1 – 15 minutes 15% B, Where mobile phases A and B consisted of 60% ACN + Buffer, and 90% IPA with 10% ACN + Buffer respectively.

Chromatograms were then processed using Agilent MassHunter Workstation and characterisation was performed for each injection by manually assessing the fragmentation spectra. PI species could easily be identified through their precursor mass and the presence of certain characteristic fragments which corresponded to the inositol headgroup [32].

3. Results and Discussion

Prior to flow injections, an initial chromatographic separation was conducted to estimate the solvent conditions under which the compounds of interest would elute, so that these conditions could be replicated as the flow injection solvent. Conditions were based off those recommended by Cajka and Feihn [21]. Briefly, a 5 uL injection of Standard solution 1 was separated over 10 minutes at 0.2mL / minute, following a gradient which ramped from 20 – 100% B using the aforementioned column which was maintained at 50 °C. Mobile phases A consisted of 60% (v/v) ACN and B 90% IPA, 10% ACN, with the addition of 10mM AmAc in each. Through this separation, it was determined that 50% B approximated the solvent conditions under which PIs would elute, thus these conditions would later be used as the flow injection mobile phase parameters. A peak corresponding to the additionally phosphorylated PI (i.e., 18:1-18:1 PI(3)P) could not be observed in this initial chromatographic separation, although at this point it was not determined as to whether this was due to an incompatibility of the chromatography or the ionisation conditions.

3.1 Buffer substitution

Flow injection experiments were conducted by substituting the buffer used, in accordance with Table 1. Through 10 replicate injections for each condition, the peak areas for the masses corresponding to 17:0-14:1 PI, 18:1-18:1 PI and 18:0-20:4 PI were recorded. In varying the concentration of each buffer, an optimal condition was determined. Whereby all data sets could then be normalised against the mean intensity of a common condition (no added buffer) to allow for direct comparison. The results shown Figure 2 are the summed intensities of all the lipids analysed.

Table 1. *Buffers and concentrations tested in flow injections.*

AmAc	AmOH	AmF
0.00 mM	0.00 mM	0.00 mM
0.25 mM	0.25 mM	0.25 mM
0.50 mM	0.50 mM	0.50 mM
1.00 mM	1.00 mM	1.00 mM
2.50 mM	2.50 mM	2.50 mM
5.00 mM		
10.00 mM		

The results in Figure 2 show that AmF and AmOH outperformed AmAc with up to a 6x increase in intensities observed in comparison to minimal changes with AmAc. It may appear that for the AmOH or AmF buffers a greater variation in signal was observed. However as shown in Figure S 1, the variations in intensity for a single species remained low, and it was the mean intensity for each individual species that drifted apart. It is suspected that with the greater ionisation efficiency observed, the slight variations in efficiency caused by saturation and chain length became more apparent, thus broadening the fold change distribution for the lipid class [28]. Whilst AmF has not previously been used as a buffer for the separation of PIs, it is known to enhance the ionisation efficiency for a broad range of compounds under both positive and negative ESI conditions [33, 34]. In these cases, the increase in signal intensity was sufficient to enable a more comprehensive level of coverage for the compounds of interest.

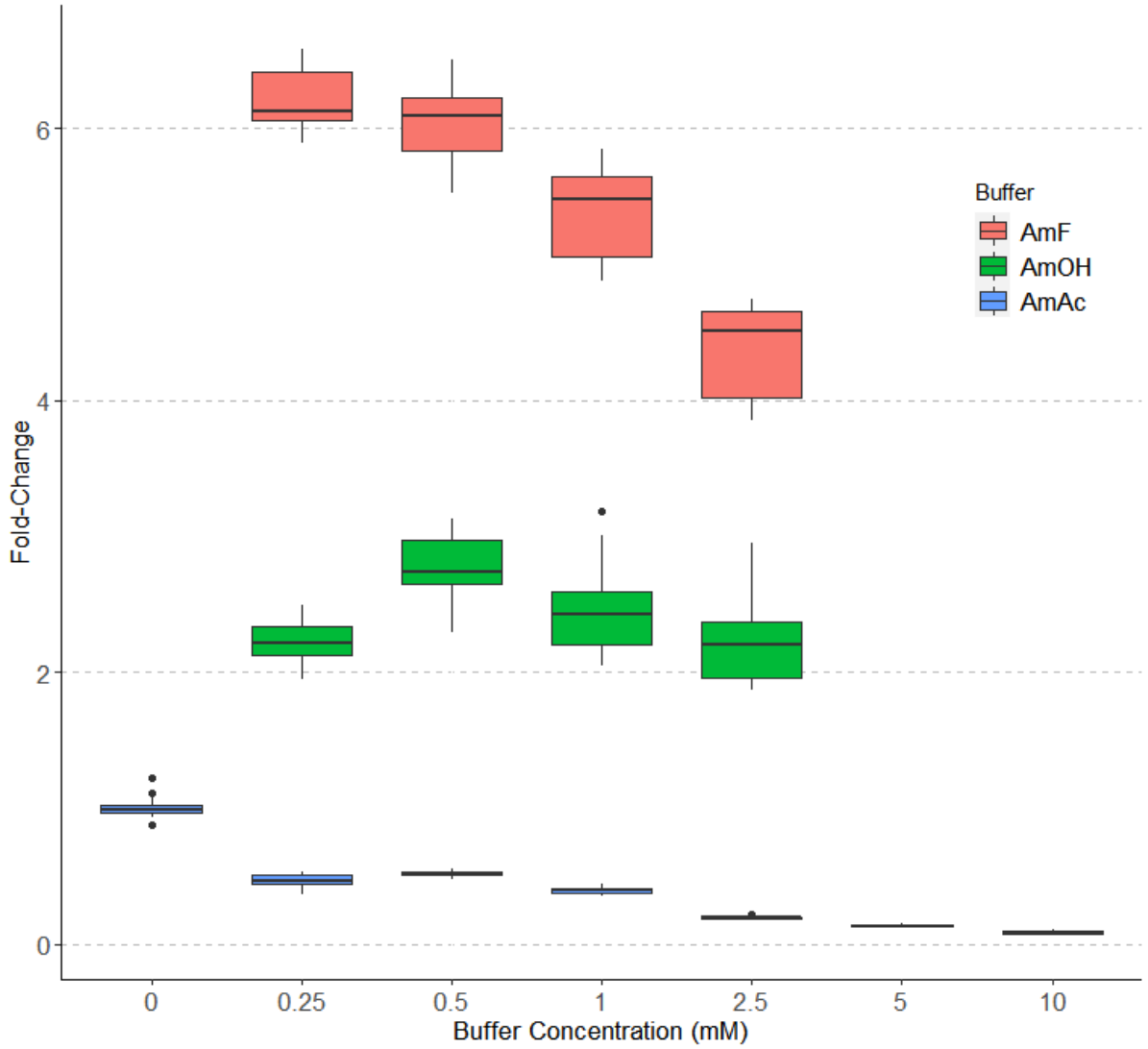

Figure 2. *Summed intensities relative to the common condition (of no added buffer), for conditions mentioned in Table 1, using an acetonitrile based mobile phase. AmF is shown in red, AmOH green,, and AmAc blue, with AmF providing the greatest increase in signal intensity.*

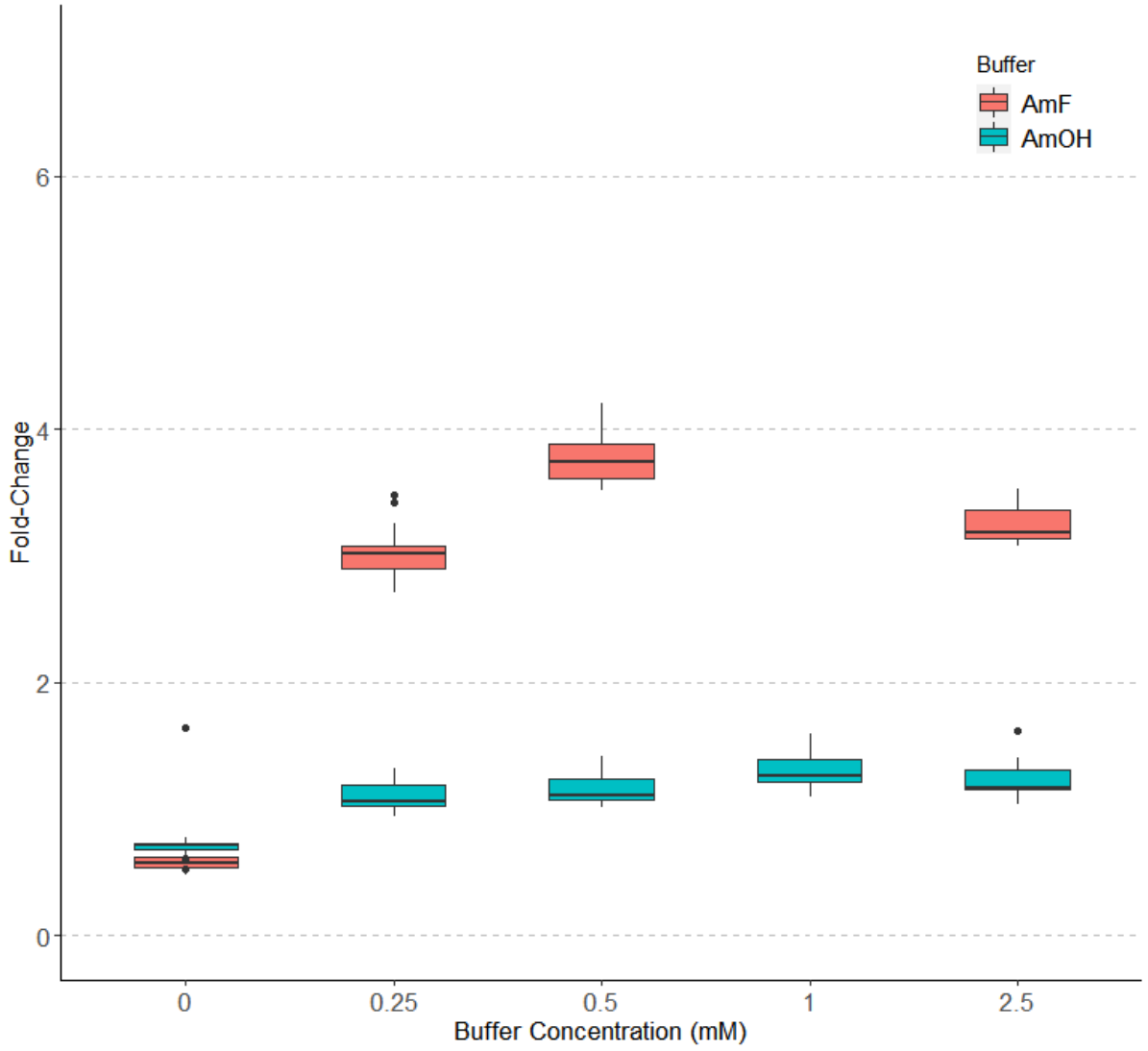

Figure 3. *Summed intensities relative to the common condition (of no added buffer), for AmF and AmOH for those conditions mentioned in Table 1, using a methanol based mobile phase. Data for the 1mM AmF experiment using the methanol based mobile phase was excluded due to an error in experimentation.*

The choice of solvent is known to have a substantial effect on the intensities of signals observed as a result of altering the ionisation efficiency for particular compounds [35]. To account for whether ACN provided favourable conditions for the ionisation of PIs, the flow injections performed using AmOH and AmF were repeated, wherein the ACN in the mobile phase was substituted with MeOH. Here, MeOH had been selected as a substitute for ACN, as it was suspected to perform similar to ACN chromatographically, while potentially providing differing conditions for ionisation due to the increased polarity of the alcohol. The results for these additional experiments may be seen in Figure 3, with the intensities not increasing in MeOH to the same degree as in ACN. Due to the unique headgroups of the individual phospholipid classes, this particular preference for an ACN based mobile phase may be particular to PIs. A similar assessment has not been completed for all lipid classes, however, contrary to the results observed here, MeOH has been shown to enhance ionisation efficiency for particular phosphorylated metabolites by several fold under negative ESI conditions, in comparison to ACN [35].

From the results shown in Figures 2 and 3, it was therefore determined that an ACN based mobile phase containing 0.25mM AmF performed best in terms of maximising MS signal intensity for the selected PI standards.

3.2 Post column additives

Table 2. *Bases and concentrations tested in flow injections. All bases were added to a mobile phase buffered with 0.25mM Ammonium Fluoride.*

Piperidine	Pyridine	NN-Diisopropylethylamine
0.00%	0.00%	0.00%
0.01%	0.01%	0.01%
0.05%	0.05%	0.05%
0.10%	0.10%	0.10%
0.20%	0.20%	0.20%

In an attempt to further enhance the observed intensity, an additional set of flow injection experiments were conducted, in which a basic modifier taken from Table 2 was added at a particular concentration. To represent the broad variety of bases used in literature, three in particular were chosen, representing an aromatic (pyridine), secondary (piperidine) and tertiary amine (*N,N*-Diisopropylethylamine). For each condition, the peak areas for the masses corresponding to 17:0-14:1 PI, 18:1-18:1 PI and 18:0-20:4 PI were recorded over 10 replicate injections and then normalised to the mean intensity for a common condition. The results of which may be seen in Figure 4 where these additives decreased the signal intensities of the PIs at all concentrations examined. This decrease in intensity appears to be contrary to the notion that an increase in pH would further facilitate deprotonation (and thus increase ionisation efficiency) and as such, may be an example of "wrong-way-round ionisation", in which more acidic conditions favour deprotonation [22]. However, having shown that an AmOH buffer somewhat favoured the ionisation of PIs (Figure 2), it is likely that the combination of AmF and the bases tested resulted in the supressed ionisation, rather than the bases themselves.

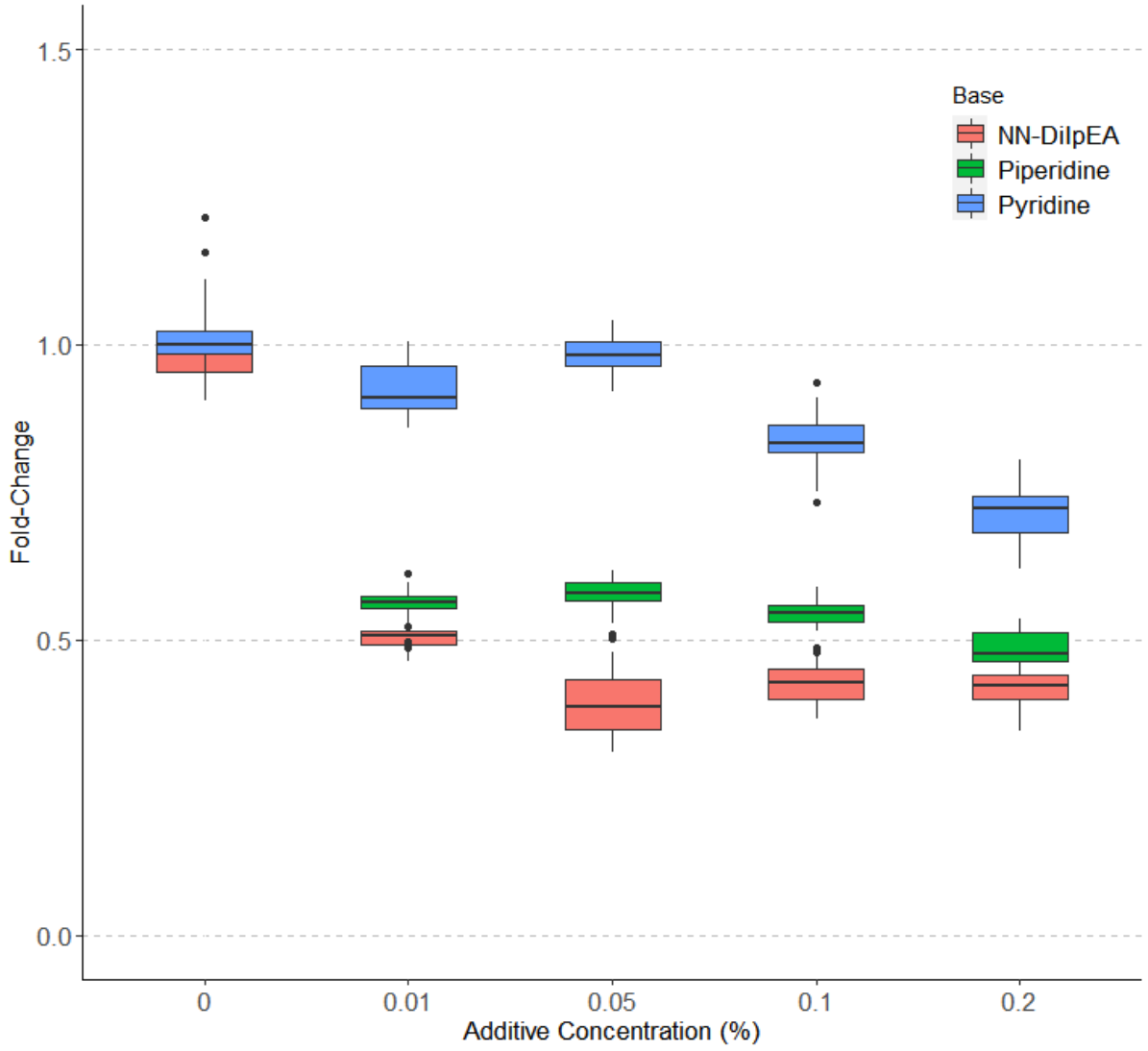

Figure 4. *Summed intensities relative to the common condition (of 0.25mM AmF with no added basic modifier), for those conditions mentioned in Table 2. NN-DilpEA is shown in red, piperidine in green, and pyridine in blue. All additives decreased the intensity at all concentrations.*

As such, through the results of these experiments it is suggested that the selected additives only served to supress the signal intensity for the observed PI standards. Therefore, the optimal conditions were determined to be that of no added basic modifier.

3.3 Chromatographic comparison

Having determined the buffer and solvent conditions for which the chosen PI standards were optimally ionised, the developed conditions were then applied to a chromatographic separation. These results could then be compared directly to those acquired using the initial 10mM AmAc modifier. Henceforth, attempted characterisations were performed on standard solution 2, which contained a dilute canola extract and internal standard. Using this standard, four replicate injections of 5uL were conducted using each condition, as mentioned previously.

For these injections, an example TIC comparing the chromatograms produced by using either buffer may be seen in Figure 5A, where it was determined that the signal intensity produced using the AmF buffer was significantly greater than that using the AmAc buffer. Furthermore, as shown in Figure 5B, a significant shift in retention time is observed for the internal standard (and thus the class as a whole,

shown in Figure 5C), eluting at approximately 5.5 minutes under AmAc to 3.5 minutes under AmF. It is believed that this shift in retention had occurred as a result of a change in mobile phase pH and may result in less resolution between the species of the class. Due to the greater intensity, the AmF chromatograms were used to identify several PI candidates through the use of their MS2 data, an example of which is shown in Figure 6. These candidates which were identified suggested a predictable relationship between the degree of desaturation, fatty acid chain length and retention time. By then plotting this relationship, the nominal masses and retention times for any additional candidates could be interpolated at the intersections of these patterns. Candidate masses which were observed at the predicted retention times, and were present though out all four replicates, suggested the validity of the identification. Although these candidates lacked corresponding MS2 data (and as such their fatty acid composition and identity could not be directly confirmed), their carbon number and degree of desaturation could be determined from their nominal mass. The completed pattern for candidates observed in the AmF separations is shown in Figure 7.

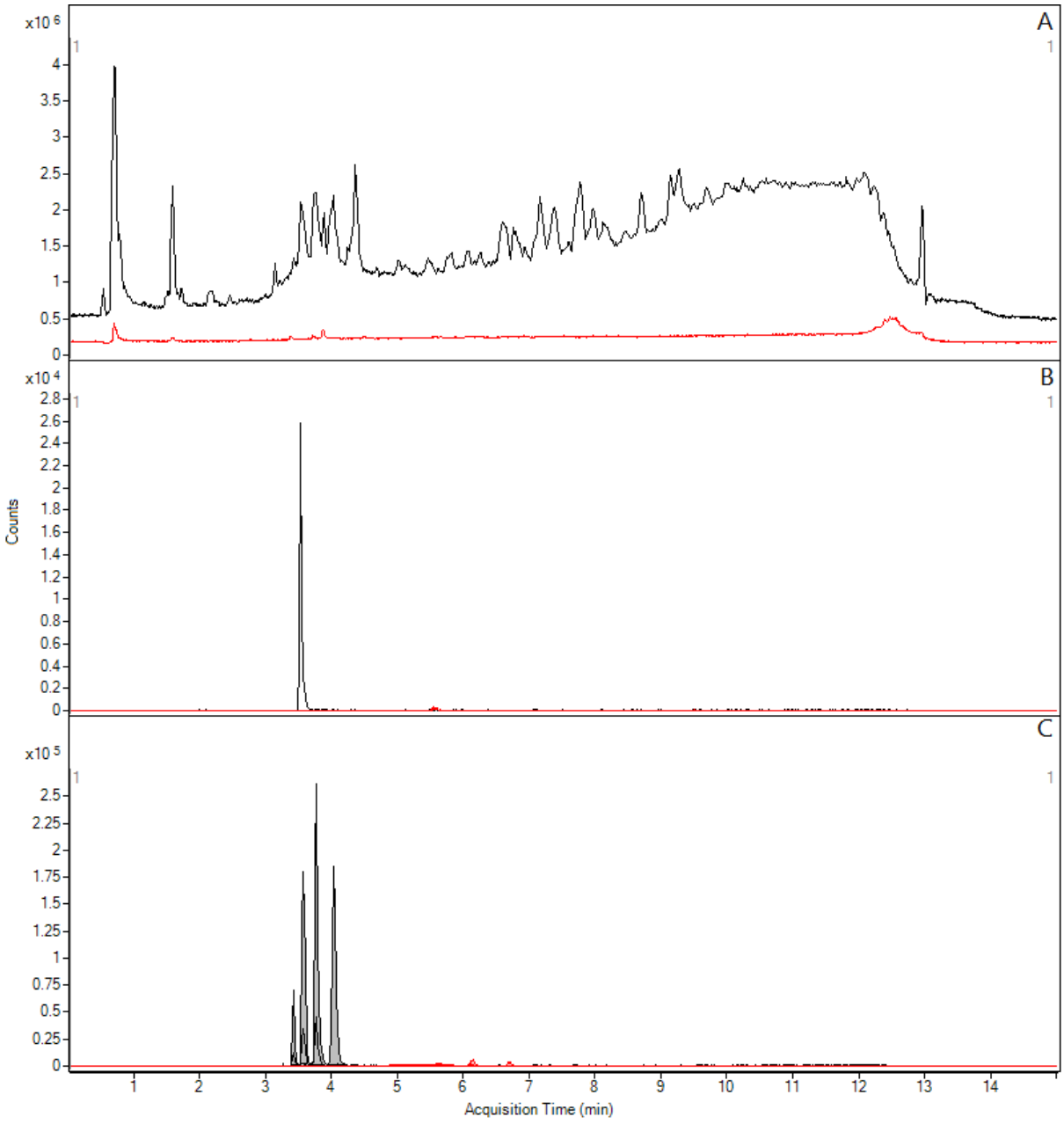

Figure 5. *Chromatograms for the AmF separations are shown in black, Chromatograms for the AmAC separation are shown in red.(A) Example TICs of standard solution 2. (B) Example EICs of*

internal standard 17:0-14:1 PI. (C) Overlayed example EICs of 4 PI candidates observed under both conditions.

No MS2 data had been gathered for the AmAc separations, due to the low intensities of the parent ions, and thus their identity had to be inferred. Both the known species and the unknown PI candidates which were observed in the AmF separation (Figure 5C) were then used to assign identity to ions of the same mass and order of elution observed in the AmAc separation. Table 3 shows the species identified under either condition, and compares the level of information which could be determined. Those species which could be observed under either condition where then used to determine the difference in signal-to-noise, shown in Figure 8. The increase in signal intensity observed with the AmF buffer did not correspond with a concurrent increase in the noise, resulting in on average a 38x increase in signal-to-noise over those obtained with the AmAc buffer.

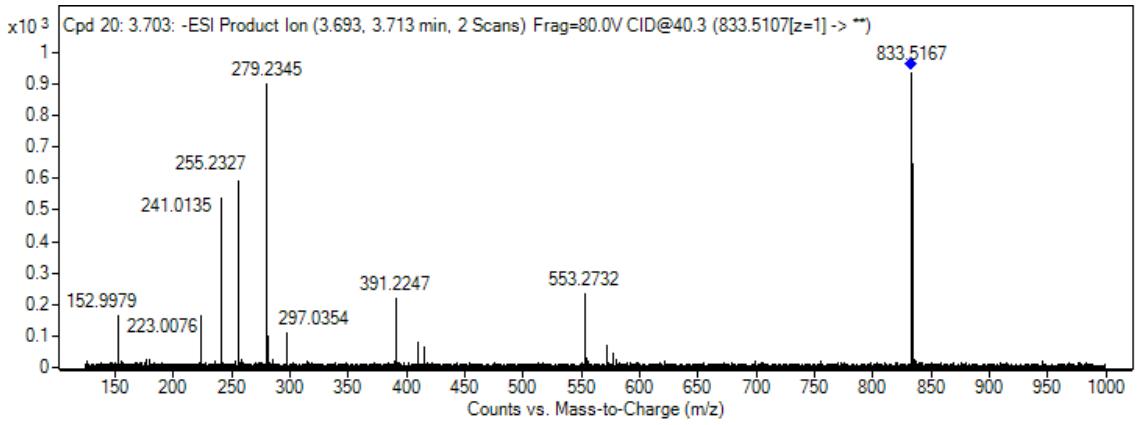

Figure 6. *Example product ion spectra of a PI candidate. m/z 153 corresponds to a fragment characteristic of glycerophospholipids, whilst m/z 223, 241 and 297 correspond to fragments characteristic of a phosphatidylinositol. Fragments m/z 255, 279, 391, 553 and 833 correspond the free fatty acids, fatty acid neutral losses and parent ion respectively.*

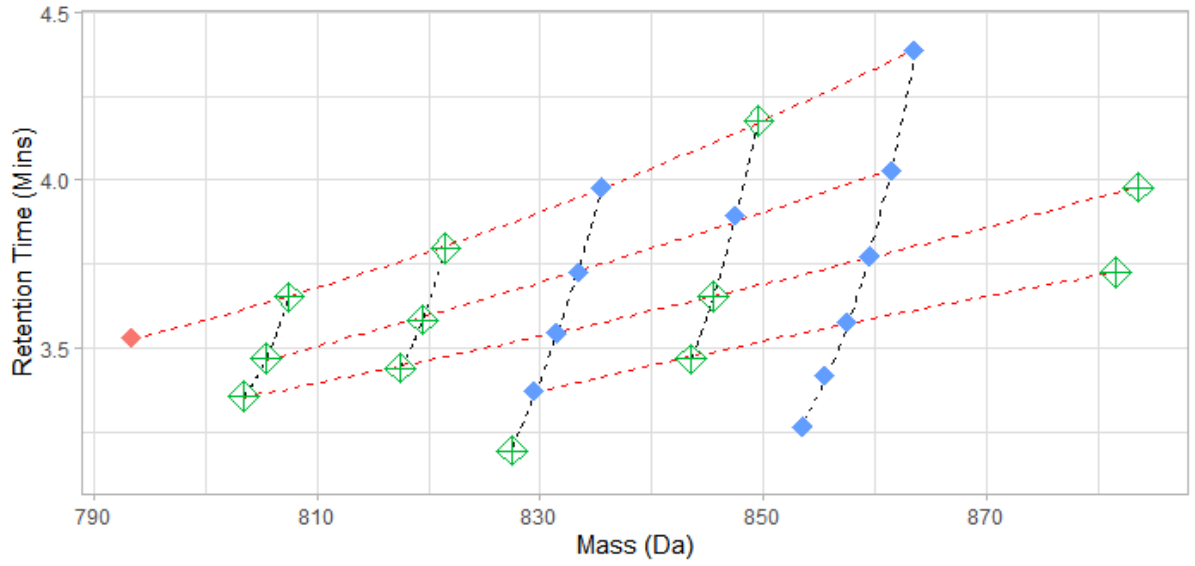

Figure 7. *PI Candidates organised by mass and retention time. The red diamond indicates the internal standard added, whereas the blue diamonds indicate PI species identified through the use of*

MS2 data. The hollow, green diamonds indicate candidates which have been proposed due to the pattern formed by identified species.

Table 3. *PI candidates observed under either condition (excluding internal standard). For those species where fatty acid composition is not specified, MS2 data had not been gathered due to low intensity, and their identity is suggested through their precursor mass.*

PI Species Observed with AmF Buffer	*PI Species Observed with AmAc Buffer*
PI 16:0_18:1	PI 34:1
PI 16:0_18:2	PI 34:2
PI 16:0_18:3, PI 16:1_18:2	PI 34:3
PI 16:2_18:2	-
PI 18:0_18:1	-
PI 18:0_18:2, PI 18:1_18:1	PI 36:2
PI 18:1_18:2	PI 36:3
PI 18:1_18:3, PI 18:2_18:2	PI 36:4
PI 18:2_18:3	-
PI 18:3_18:3	-
PI 32:1	-
PI 32:2	-
PI 32:3	-
PI 33:1	-
PI 33:2	-
PI 33:3	-
PI 34:5	-
PI 35:1	-
PI 17:0_18:2	-
PI 35:3	-
PI 35:4	-
PI 38:5	-
PI 38:6	-

In total, through the use of the AmF buffer, 14 PI species were identified, and 12 PI candidates are suggested; whereas with the AmAc buffer, 0 PI species could be identified and only 6 candidates are suggested. All species observed using the AmF buffer, with their intensities relative to the internal standard, are shown in Figure 9.

From this data it is apparent that there is a broad distribution of minor PI species within the canola extract, whereas only a few contribute to the bulk of the class. As result of the disparity in terms of signal intensity between the newly developed and previously reported conditions, it is clear that this molecular diversity could not have been observed using previously applied methods from the literature. Previous comprehensive characterisations of plant phospholipids lack the same depth of analysis in terms of coverage of PI species, despite employing either highly concentrated samples or more sensitive instrumentation. For example, one such analysis targeting PIs observed only 10 species in an extract from 20mg of infant formula, concentrated into an unspecified volume [10]. In cases of general phospholipid characterisations, one analysis observed 7 PI species in an extract from 2.5g flax seed concentrated into 1mL [36], a second observed only 3 PI species in an extract from 0.5g of wheat roots concentrated into 1mL [37], a third observed 25 PI species in a variety of nuts from 0.5g concentrated into 0.5mL [38], and a fourth observed 12 PI species in a variety of sunflower seeds from up to 0.5g concentrated into an unspecified volume after a series of extractions and digestions [39]. In comparison, this procedure observed 26 PI species in an extract from 0.1g canola seed, concentrated into 2mL, and then diluted to half its concentration.

Of those observed, many of the more intense PI candidates shown in Figure 9 constitute more than one co-eluted isobaric species. Alike all phospholipids, PIs are known to contain a number of double bond and positional isomers which are difficult to separate under standard reverse phase conditions [20]. Therefore, it is suggested that further chromatographic development or the use of ion mobility spectrometry is required to interrogate these isomeric species.

Using those conditions which were previously suggested in literature (i.e. the 10mM AmAc buffer suggested by Cajka and Feihn), chromatographically separating these isobars and isomers would have proved impractical as the already minor peaks would be further diminished in intensity as each previously co-eluting species is resolved. However, it is apparent that under these newly proposed conditions (i.e. 0.25mM AmF), the opportunity for further chromatographic resolution, and thus a more comprehensive level of characterisation, is available for future consideration.

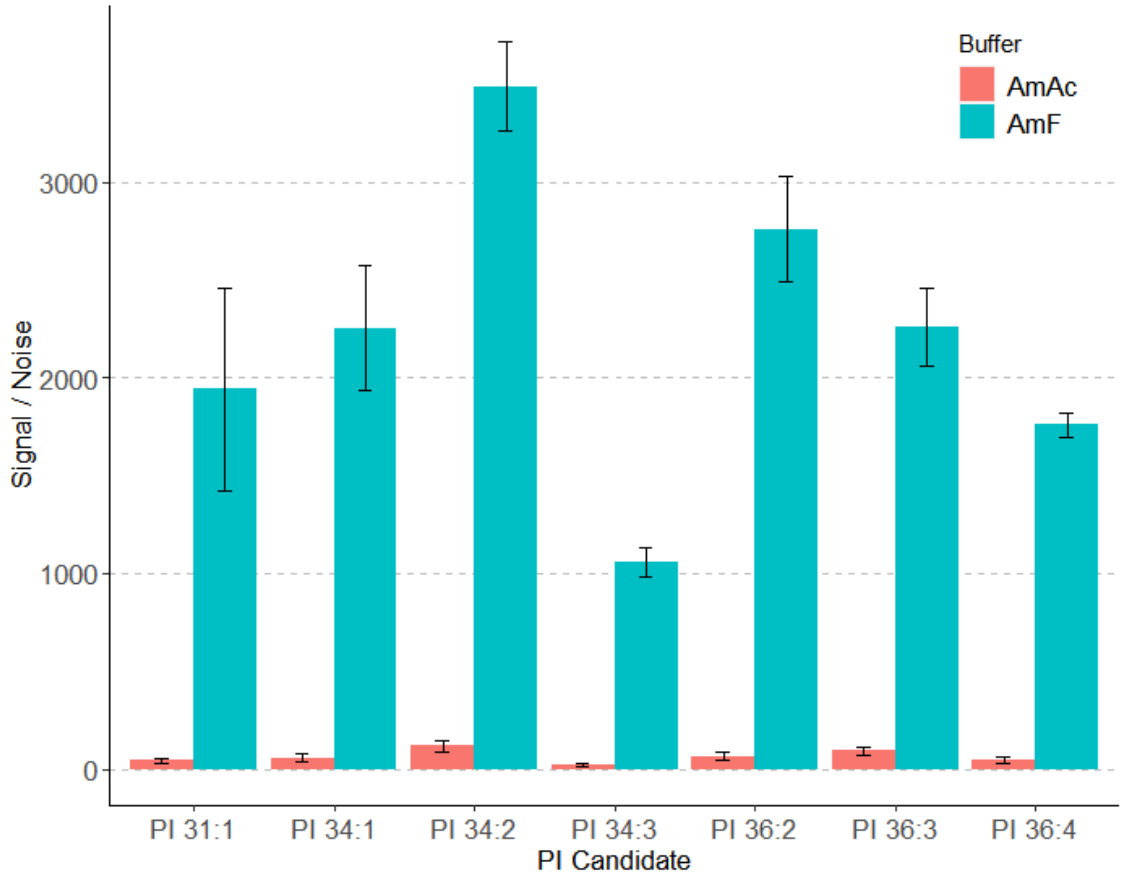

Figure 8. *Signal-to-Noise ratio of PI species observed under both conditions. Values were calculated based on peak height and RMS of noise intensity. On average the signal to noise ratio produced for each species using the AmF buffer was 38-fold greater than that produced using the AmAc buffer.*

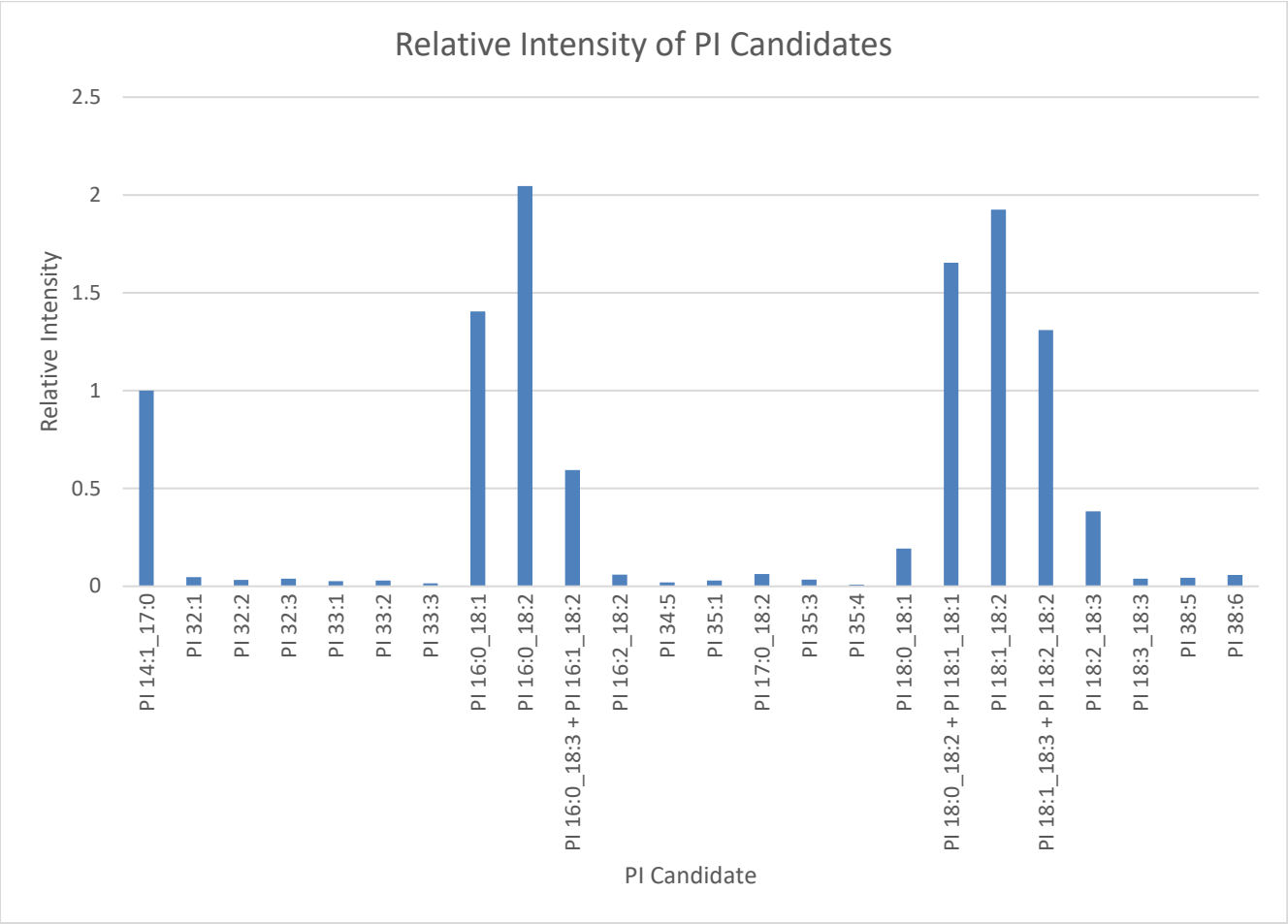

Figure 9. *All PI species observed in the canola extract with their intensity relative to the internal standard PI 14:1 17:0. From the distribution, it is apparent that a large proportion of the species are relatively non-abundant. Furthermore, it may be seen that several of the more intensely observed PI species are composed of more than one isobar.*

4. Conclusion

In summary, it is suggested that the conditions generalised for lipidomic analysis are in particular insufficient for the analysis of phosphatidylinositol (PI) species, and it is proposed that a more comprehensive characterisation of PI species may be conducted by biasing the generalised conditions as to favour their ionisation. Experimentation revealed that out of those conditions tested, a mobile phase based on ACN, containing 0.25mM AmF, sans modifier, significantly outperforms previously suggested 10mM AmAc, sans modifier, mobile phase for the purpose of maximising PI ionisation efficiency.

To demonstrate the utility of these newly proposed mobile phase conditions, a characterisation of PIs extracted from canola seed was performed using both sets of conditions, through which it is apparent that the newly developed conditions has enabled a more comprehensive analysis, and revealed the diversity of minor PI species present. Furthermore, it is suggested that these newly proposed conditions provide the opportunity to further enhance chromatographic separation, where previously it may have been limited due to the low ionisation efficiency.

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5. Supplementary Data

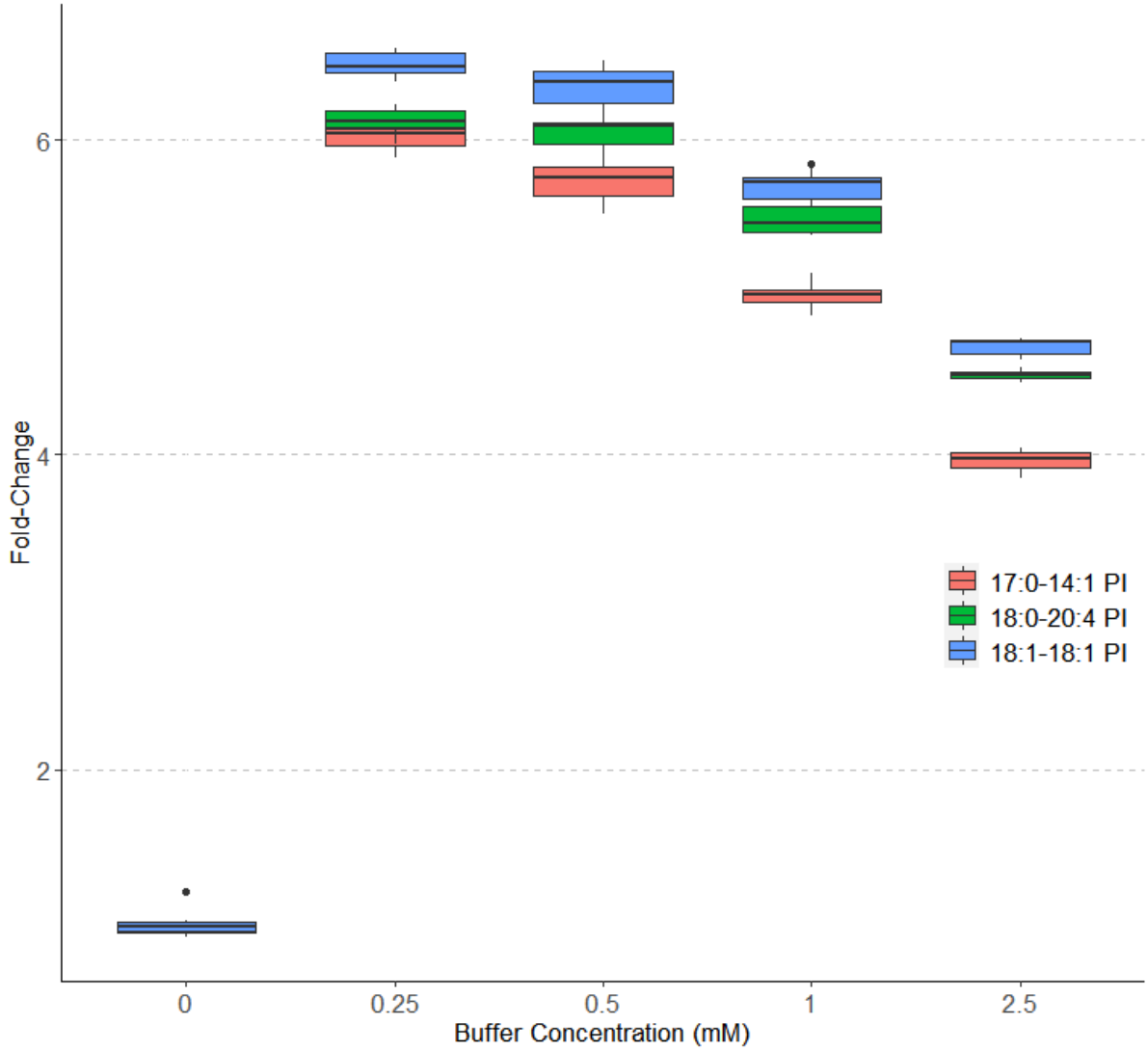

Figure S 1. *Intensities of the individual PI species under AmF*